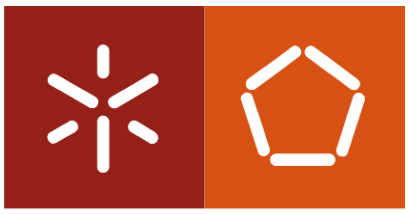
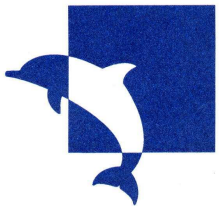


UNIVERSITY OF MINHO



UNIVERSITY OF SOUTHAMPTON

Survival of *Helicobacter pylori* in drinking water and associated biofilms

Dissertation for PhD degree in Chemical and Biological Engineering

SUPERVISOR: Prof. M. J. Vieira

CO-SUPERVISOR: Prof. C. W. Keevil

NUNO FILIPE RIBEIRO PINTO DE OLIVEIRA AZEVEDO

Acknowledgements

I would like to thank my supervisor Professor M. J. Vieira and co-supervisor Professor C. W. Keevil for their encouragement and kind advice throughout my PhD studies. It has been a privilege and an honour, not only because of the scientific guidance provided, but also for the moments of leisure shared together.

I also would like to thank to my colleagues at the Center of Biological Engineering of the University of Minho (Dr. Rosário Oliveira, Dr. Joana Azeredo, Olívia, Pilar, Mariana, Salomé, Sanna, Manuel, Nuno Cerca, Nuno Reis, Lívia, Ricardo, Ana Lobo, Cristina) and those who have worked with me more closely (Ana Paula, Ana Rita, Lúcia and Sofia) for providing a pleasurable and friendly working atmosphere. It would be difficult to find better persons to work with.

For their hospitality and support, I'm grateful to the Environmental Healthcare Unit in Southampton, especially Sandra, Sarah, Ian, Jonathan, Danny, Lucy, Chris and Matt. In such good company it's easier to spend time away from home.

Because work means nothing without leisure, I express my gratitude to the friends who have shared with me moments of joy throughout these years.

Last but certainly not least, I would like to thank my family and my parents, Alfredo and Justina, as well as my girlfriend Sónia, for always being there for me even though large distances separated us at times. They were the ones that most influenced my development as an individual and I will always be indebted to them.

Resumo

O *Helicobacter pylori* é uma bactéria Gram-negativa taxonomicamente relacionada com *Campylobacter* spp. Embora a maioria das pessoas infectadas com *H. pylori* sejam assintomáticas, a bactéria tem sido implicada no desenvolvimento de certas doenças, tais como as úlceras gástricas e os cancros do estômago. Nos últimos 10 anos, o *H. pylori* tornou-se numa das bactérias mais estudadas pela comunidade científica. A investigação efectuada permitiu caracterizar muitos dos aspectos fisiológicos da bactéria, assim como determinar a sua prevalência na população humana em diferentes países e clarificar o seu papel no desenvolvimento de certas doenças. No entanto, a rota (ou rotas) de transmissão entre a população humana ainda não foram identificadas. A falta desta informação assume particular relevância em Portugal, um país onde estudos epidemiológicos indicam que a prevalência do *H. pylori* é uma das maiores entre os países da Comunidade Europeia.

Neste trabalho foram efectuados estudos que pretendem clarificar o papel da água como meio de transmissão do *H. pylori*, dando especial relevância aos biofilmes formados na superfície do material das tubagens, onde a diversidade de microambientes criados pelo consórcio de bactérias pode fornecer um ambiente mais propício para a sobrevivência desta bactéria. Para tal, foi desenvolvida uma nova sonda molecular, baseada em PNA, que permite a identificação *in situ* do microrganismo neste tipo de consórcios. A aplicação desta técnica em biofilmes formados em laboratório demonstrou que a bactéria parece ter tendência para se localizar na base das estruturas dos biofilme onde podem existir zonas de microaerofília que podem prolongar a sua sobrevivência.

Um estudo sobre a influência de diferentes parâmetros ambientais na adesão da bactéria também foi realizado. O estudo indicou a ausência de forcas hidrodinâmicas como o factor determinante na obtenção de uma maior adesão do *H. pylori* a superfícies, indicando os biofilmes formados em água do poço como locais preferenciais para a detecção da bactéria. A coloração da bactéria aderida com fluorocromos indicativos de viabilidade demonstrou, no entanto, que o *H. pylori* se começava a tornar não viável após cerca de 48 horas, indicando que a co-agregação com outras espécies pode ser necessária para a sobrevivência da bactéria.

Após ter sido provado neste trabalho que o *H. pylori* sofria um choque de nutrientes quando era recuperada da água para meios de cultura com alto teor de nutrientes, um novo meio com baixo teor em nutrientes foi desenvolvido. O meio causou uma melhoria de mais de 30% na recuperação da bactéria para três das oito estirpes analisadas. A associação deste meio com substâncias selectivas para a bactéria publicadas na literatura causa no entanto uma pequena diminuição na eficiência.

A aplicação das novas técnicas de biologia molecular e meios de cultura a sistemas reais não permitiu uma identificação positiva consistente da bactéria em amostras de água ou biofilmes. Parte deste insucesso pode dever-se no entanto à falta de aperfeiçoamento das técnicas para sistemas reais, assim como ao limitado número de amostras analisado. A procura de uma evidência que prove indubitavelmente que a água pode ser um veículo de infecção continua assim em aberto, embora a trabalho realizado neste projecto indique que este tipo de sistemas possa efectivamente conter *H. pylori* viável, trazendo obviamente preocupações para as entidades responsaveis pela saúde pública.

Abstract

Helicobacter pylori is a Gram-negative bacterium closely related to *Campylobacter* spp. Although most people who are infected with *H. pylori* are asymptomatic, the bacterium has been implicated in the development of certain diseases, such as gastric ulcers and stomach cancers. In the last 10 years, *H. pylori* has become one of the hot-topics for scientists who have managed to discover many aspects of the bacterium's physiology, as well as describe its prevalence in the human population in several countries all around the world and clarify its role in certain human diseases. However, the route (or routes) of transmission among the human population are yet to be identified. This lack of information assumes particular importance to Portugal, a country where the data available indicates that *H. pylori* prevalence ranks amongst the highest in the European Community.

With this work it was attempted to clarify the role of water as a means of transmission of *H. pylori*, with special emphasis being given to biofilms formed on pipe surfaces, where the diversity of microenvironments created by the heterotrophic consortium of bacteria could provide a more suitable environment for the survival of the pathogen. As such, a new molecular probe based on peptide nucleic acids was designed, which allow the *in situ* identification in this type of consortia. The application of this technique in lab-formed biofilms has shown that *H. pylori* subsists close to the basal layer of the biofilm, which suggests that the bacterium is able to migrate to low redox zones created by these structures to enhance survival.

The influence of different environmental parameters on *H. pylori* adhesion was also studied. The bacteria appeared to adhere in higher numbers in the absence of shear forces, indicating biofilms formed on the surfaces exposed to well-water as preferential places for the location of the bacteria. After viability-staining of the adhered pathogen, it was observed that *H. pylori* would start to become non-viable after 48 hours, which indicates that co-aggregation with other species might be necessary for the survival of the bacteria.

After being proved in this work that a nutrient shock occurred in *H. pylori* when it was recovered from water to a nutrient-rich media, a new low-nutrient medium was developed. The medium has caused an improvement of recovery of more than 30% for three out of

the eight strains tested. The addition of antibiotics already described in the literature to this new medium causes however a decrease in this efficiency.

The application of new methods of molecular biology and plating techniques to field studies has not allowed for an unequivocal positive identification of *H. pylori* in water or water-associated biofilm samples. Part of this failure may be attributed to the lack of suitability of these techniques to real systems, as well as to the limited number of samples processed. The search for evidence that unquestionably proves that water might be a vehicle of infection therefore remains active. Nevertheless, the work performed so far indicates that these types of systems are able to support the existence of viable *H. pylori*, and therefore be a cause for public health concern.

Contents

List of Figures

List of Tables

1 General introduction

Helicobacter pylori is a Gram-negative bacterium closely related to *Campylobacter* spp. Although most people who are infected with *H. pylori* are asymptomatic, the bacterium has been implicated in the development of certain diseases, such as gastric or duodenal ulcers, and stomach cancers. At present, the route of transmission is perhaps one of the most controversial areas of *H. pylori* research. Several reservoirs outside the human gastrointestinal tract have been suggested as sources of infection, but evidence is not conclusive. The main goal of this thesis addresses part of this problem, by trying to establish the role of water and drinking water biofilms in *H. pylori* transmission.

This Chapter presents general features of *H. pylori*, such as a brief historical introduction in the next section and physiological and morphological characteristics in Section 1.3. Then follows an overview of the clinical relevance of the bacterium and occurrence status in populations worldwide. A special focus on the suspected transmission routes between human populations is presented in Section 1.5, and the last section addresses biofilms and their relevance in the harboring of important pathogens.

1.1 The emergence of *Helicobacter pylori*

Since the late 19th century, European pathologists have noticed the presence of curved bacterial cells in gastric biopsy specimens submitted to histological examination (146). However, the first work reporting the plate culture of *H. pylori* was only published in 1984 by two Australian scientists, Warren and Marshall (148). In this study, *H. pylori* was isolated from biopsy specimens taken from intact areas of the antral mucosa of human patients and, because it was present in nearly all individuals with active chronic gastritis, duodenal ulcer, or gastric ulcer, it was considered to be an important factor in the aetiology of these diseases. The morphological and physiological similarities between this new microorganism and the *Campylobacter* genus, lead scientists to firstly name it as *Campylobacter pyloridis* (225), which was shortly after corrected to *Campylobacter pylori* (147). The appearance of *H. pylori* current denomination occurred in 1989 (75), after important physiological differences between this organism and other *Campylobacter* spp. were identified.

Since those early days, many advances in the understanding of *H. pylori* particular characteristics were achieved. A great deal of the bacterium's biochemical pathways has been identified, the prevalence in the human population in several countries all around the world described, and its role in certain human diseases such as gastritis and stomach ulcer clarified. Research has also allowed for the development of reliable diagnostic methods for *H. pylori* infection and suitable treatment procedures. Many questions, however, remain. For instance, the role of the bacteria in other diseases is still under great debate, as well as the route of transmission between the human population.

The peculiarity of *H. pylori* has also turned the bacteria into one of the hot-topics for scientists. Between 2000 and 2004, an average of ≈ 1500 papers were published annually in international peer-reviewed journals, only 600 less than *Escherichia coli* and over 1000 more than *Salmonella typhimurium* (Fig. 1.1). It was also one of the first bacteria to have the genome sequenced for two different strains, J99 (2) and 26695 (238).

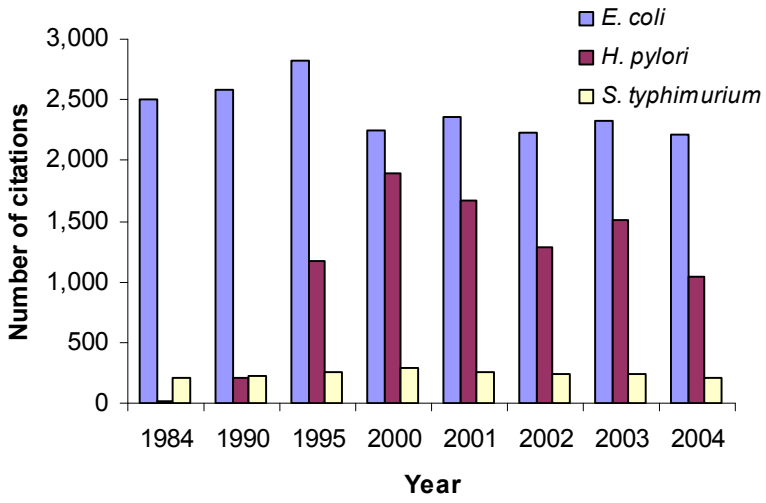


Fig. 1.1. Progression in the number of papers published at international journals for three different microorganisms with time, as obtained using the ISI Web of Knowledge search engine (http://isi4.newisiknowledge.com/portal.cgi/wos). The search was performed with the words "Helicobacter AND pylori", "Escherichia AND coli" and "Salmonella AND typhimurium" in the title field. In 1984 and 1990, the result of the search for *H. pylori* was added to the results obtained with "Campylobacter AND pylori" and "Campylobacter AND pyloridis".

H. pylori was the precursor for the identification of a large number of related microorganisms inhabiting other mammals and birds gastrointestinal (GI) tract. By 2001, there were 20 formally named species comprising the genus *Helicobacter* (226), and new *Helicobacter* species are now regularly being discovered (e.g. 83, 84, 264). Most of the members do not normally colonize the gastric mucosa, but instead thrive in the intestinal tract and/or the liver of the host and are therefore classified as enterohepatic (217). Those inhabiting the stomach, such as *H. pylori*, are termed gastric (49). So far, only *H. pylori* has been found to generally inhabit humans, even though *H. heilmannii* has been occasionally detected.

1.2 General aspects of *H. pylori*

1.2.1 Taxonomy

Included in the group of Gram-negative bacteria, *H. pylori* belongs to the phylum of proteobacteria, which is the largest and most physiologically diverse of all Bacteria, comprising members of the normal flora of animals (e.g. *E. coli, Neisseria*), as well as a wide variety of pathogens of animals (e.g. *Rickettsia, Salmonella, Vibrio*) and plants (e.g. *Agrobacterium tumefaciens, Burkholderia*) (142). Based on analysis of their 16S ribosomal RNA, *Helicobacter* is sub-categorized into the epsilon proteobacteria class, which includes other genera such as *Thiovulum*, *Camplylobacter, Sulfurospirillum*, *Wolinella*, and *Arcobacter*, among others. This group of bacteria inhabits very different environments, from aquatic habitats (*Sulfurospirillum* spp.) to mucosal surfaces of the gastrointestinal and/or urogenital tract of bird and animals (*Campylobacter* spp. and *H. pylori*). They are all spiral-shaped at some point in their life cycles, multiply by binary fission, and able to grow microaerobically or anaerobically.

1.2.2 Metabolism and physiology

As with all bacteria, *H. pylori* uses adenosine triphosphate (ATP) to conserve energy and perform energy demanding reactions. To generate the necessary ATP molecules, the microorganism creates a proton electrochemical gradient across the cytoplasmic membrane. In *H. pylori*, the prime generator of this gradient is the respiratory chain, where organic, such as D-glucose and formate, or inorganic, such as H_2 (179), compounds are submitted to a process of oxidation. The only known electron acceptors are O_2 and possibly fumarate (126, 145), explaining the obligate requirement for O_2 in this bacterium. Conversely, *H. pylori* possesses several essential metabolic enzymes that are typical of anaerobic-type metabolism, and therefore oxygen-labile (115). Taken together, these factors may partly account for the microaerophilic character of the bacteria, despite the fact that it possesses several oxidative stress response systems (86). Growth of the microorganism *in vitro* occurs therefore at low concentrations of oxygen (<10%), which

has been suggested to also approximate oxygen tensions found in the gastric mucosa (115).

In terms of the metabolic aspects of carbon flow in *H. pylori*, there is strong evidence, both from genomic and biochemical analysis, supporting the presence of a standard Embden-Meyerhof pathway, which degrades glucose to pyruvate (92). However, the citric acid cycle (CAC), another major pathway in respiration, is known to be quite unique. This cycle usually plays a key role both in catabolic and biosynthetic pathways and is present in most bacteria. Genomic analysis failed to identify homologs of several genes encoding enzymes necessary to the typical CAC and it has therefore been suggested that the bacterium possesses a branched incomplete CAC (114, 145). Detailed description of other biosynthetic pathways can be found elsewhere for fatty acids (114), amino acids (50), nucleotides (156) and peptidoglycan (122).

To survive and replicate in the extreme environment of the human stomach, *H. pylori* developed certain features that are quite exceptional in the bacterial world. One of such features is an enzyme called urease, which catalyzes the hydrolysis of urea into carbon dioxide and ammonia thus conferring to the bacteria extended acid resistence (Fig. 1.2). Another strategy to avoid the acid environment of the human stomach is the spiral shape of *H. pylori* and the seven sheathed flagella present in one of its poles. It allows the bacterium to be highly motile even in very viscous mucus, allowing it to migrate to the mucus layer overlaying the gastric mucosa (4). This mucus is relatively thick and viscous and maintains a pH gradient from approximately pH 2 adjacent to the gastric lumen to pH 7.4 immediately above the epithelial cells (11). Adaptation to the stomach has also probably played a major role in the absence of some amino acid synthesis pathways. The bacterium is therefore dependent on many of the host's amino acids, for which it has transporter systems, for the transcriptional apparatus. These amino acids may also serve as sources of carbon. The adaptation of *H. pylori* to the human stomach has turned it into a very fastidious microorganism, requiring a complex medium and a few days to be grown *in vitro*.

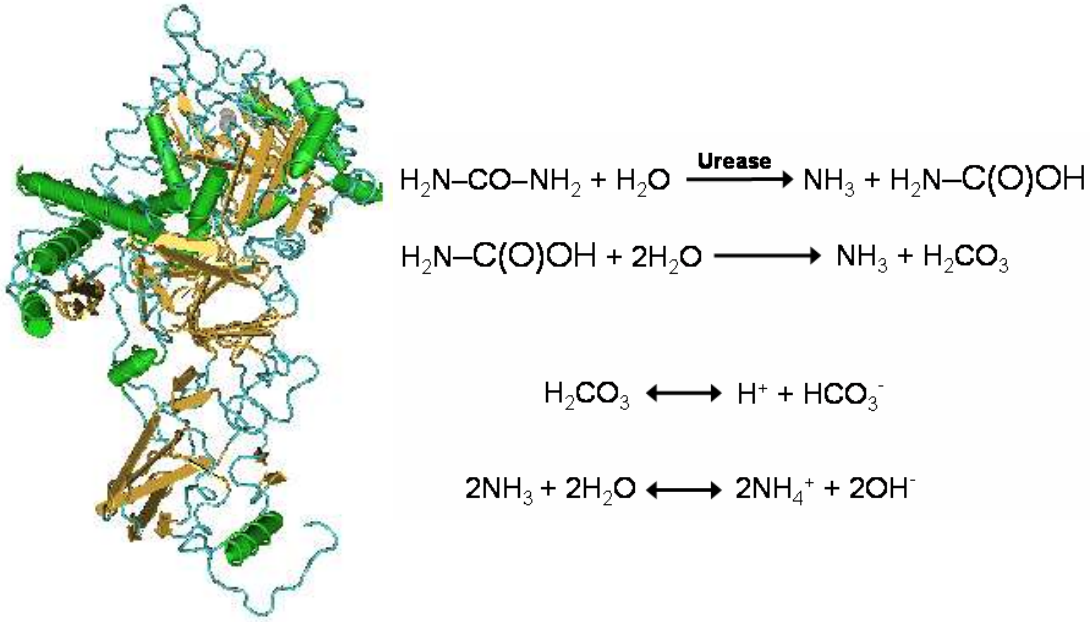


Fig. 1.2. The *H. pylori* urease enzyme structure and the chemical reaction it catalyzes. In aqueous solutions, the released carbonic acid and the two molecules of ammonia cause an increase in pH. The structure was obtained from the program CND3 (freely available at the NCBI website).

1.2.3 Morphology

Although the spiral shape was the only morphology originally described and the most commonly found *in vivo*, it is now known that this pathogen can also assume an alternative coccoid form, passing through an intermediate U-shape during the conversion from one form to another (4). The spiral form of *H. pylori* is a curved rod that is generally 2 to 4 μm long and 0.5 to 0.8 μm wide, while coccoid forms range from 1 to 4 μm. *H. pylori* can also be grown in a longer spiral form (approx. 20 μm long) under certain growth conditions *in vitro*, resembling that described for *H. heilmannii* (252).

The significance of each of the morphological types of *H. pylori* in physiological mechanisms is uncertain, and a correct understanding of the functionality and infectivity potential of each of them is essential for the interpretation of many of the results obtained throughout this thesis. Because the spiral shape is commonly found *in vivo*, it has long

been associated with the infectious form of the pathogen. Transformation to the coccoid form can be induced by exposing the microorganism to sub-optimal conditions, such as nutrient deprivation (261), and prolonged incubation (164), suggesting that these forms are a dormant stage of *H. pylori* and might play a role in the survival of the bacterium in a hostile environment. Other authors argue, however, that conversion of the pathogen from the spiral to the coccoid form is a passive process that does not require protein synthesis, and hence defend that this later form represents inactive "dead" cells (29, 124). Saito *et al*. have classified coccoid forms of *H. pylori* into three types representing plural transformation-processes from the spiral morphology: the dying coccoid forms, the living ones with culturability and the viable but non-culturable ones (213).

1.2.4 Genome

The genome of *H. pylori* contains ≈ 1.7 Megabase pairs (about one-third that of *E. coli*), with a G+C content of 39% and ≈ 1,500 predicted coding sequences (238). Comparison between the two complete genomic sequences of *H. pylori* strains demonstrated that even though the chromosomes are organized differently in a limited number of discrete regions, the genome size, genetic content, and gene order of these two strains are very similar (2). Between 6 to 7% of the genes are specific to each strain, with almost half of these genes being clustered in a single hypervariable region (3). There is, however, a remarkable diversity expressed by *H. pylori* strains (70, 214). For the generation of such diversity, *H. pylori* uses typical bacterial mechanisms that involve endogenous point mutations (14), intragenomic recombination (102, 197, 238) and niche sectoring (99). These traits may be viewed as evidence of a versatile population, able to maximize resource utilization in a variety of niches and microniches and to avoid host constraints (15).

1.3 Clinical relevance of *H. pylori* and worldwide prevalence

When *H. pylori* colonizes the ecological niche of the human stomach, a precarious balance is established between the bacterium and the host. Most of the human population will carry the microorganism asymptomatically, and even when detected, it is usual to leave the subject untreated. If left untreated, *H. pylori* infection will persist in the human

host, although in the early years of life, natural clearance of the bacterium may occur (158). More research is needed, but randomized controlled trial data recommend treatment regardless of symptoms because of the increased risk of gastric cancer in the untreated individual (160). *H. pylori* also has a major role in promoting risk of peptic and duodenal ulcer disease and gastric MALT lymphoma, but emerging evidence suggests that gastric *H. pylori* colonization has a protective role in relation to severe gastro-esophageal reflux disease and its sequelae, Barrett esophagus and adenocarcinoma of the esophagus (15). The role of the infection in other human conditions, such as cardiovascular disease (27) and obesity (234), remains inconclusive.

After the implication of *H. pylori* in human disease, eradication therapies have evolved from cumbersome and sometimes ineffective month-long regimens to the triple therapy that is now applied worldwide. Triple therapy is a 1 or 2-week course of treatment which involves taking two antibiotics (e.g. metronidazole, tetracycline, amoxicillin) and either an acid suppressor (e.g. cimetidine, omeprazole) or a stomach lining shield (usually bismuth subsalicylate) (269). In adults, triple therapy reduces ulcer symptoms and prevents ulcer recurrence in more than 90 percent of patients (222), however it still involves taking as many as 20 pills a day and may cause mild side effects such as nausea, vomiting and diarrhea. Furthermore, the improved resistance of the bacterium to antibiotics, as well as the poor patient compliance are causing an increased failure of this *H. pylori* eradication therapy (153). Alternative therapies, involving different time regimens and quantity of antibiotics (e.g. quadruple therapy), and new antibiotics or probiotics, such as lactoferrin, ginger root extracts and *Lactobacillus gasseri*, are therefore being evaluated (153, 222).

An aspect that has also deserved particular attention in the last few years is the identification of virulence factors. Some of them, such as urease, are obviously involved in the survival of the bacteria in the human stomach and have been described in Section 1.2.2. Others, such as the cytotoxin-associated antigen (CagA), the vacuolating toxin (VacA) and the adherence factor BabA, have been detected at a higher frequency in strains of *H. pylori* infecting humans that have developed *H. pylori*-associated pathologies when compared with strains infecting asymptomatic humans (198). Virulence factors are evident vaccine candidates and some have already been or are being tested in humans as a

potential vaccine (209). To more effectively achieve this goal, research interest is now shifting from the descriptive association of virulence factors with clinical outcome in infected patients to the molecular mechanisms of virulence factor action.

Presence of *H. pylori* can be detected using invasive or non invasive detection techniques. Invasive techniques (biopsies) include the urease test, which detects the presence of this important *H. pylori* enzyme and allows a rapid presumptive diagnosis. Definitive diagnosis can be then achieved either by staining of histological sections of the stomach lining or by standard culture methods. The non-invasive methods include the breath test, where urea is swallowed and, if *H. pylori* is present, broken down into carbon dioxide which is then exhaled and can be measured in the breath. Also, serological tests that measure antibodies against the pathogen are available. A positive antibody test can either stand for an active infection, or be a sign that infection was present in the past and is now cleared. The choice of test is determined by clinical indications, pretest probability of infection, as well as the availability, cost, sensitivity and specificity (180).

H. pylori infection occurs worldwide, but significant differences in the prevalence have been found both within and between countries (158). Generally, the overall prevalence is higher in the countries of underdeveloped regions, such as Africa and Asia, than in the more developed countries in Western Europe and North America (Fig. 1.3). Perhaps the most notable exceptions for this trend are Japan and Portugal, where prevalence of *H. pylori* is higher than 80%, despite the fact that they are ranked 9th and 26th in the Human Development Index compiled by the United Nations Development programme (66). Overall, *H. pylori* prevalence appears to be decreasing as a result of improved sanitary conditions and treatment procedures. It is now well known that *H. pylori* infection is mostly acquired in childhood, and that by the age of 10 more than 50% of children worldwide carry this organism (196).

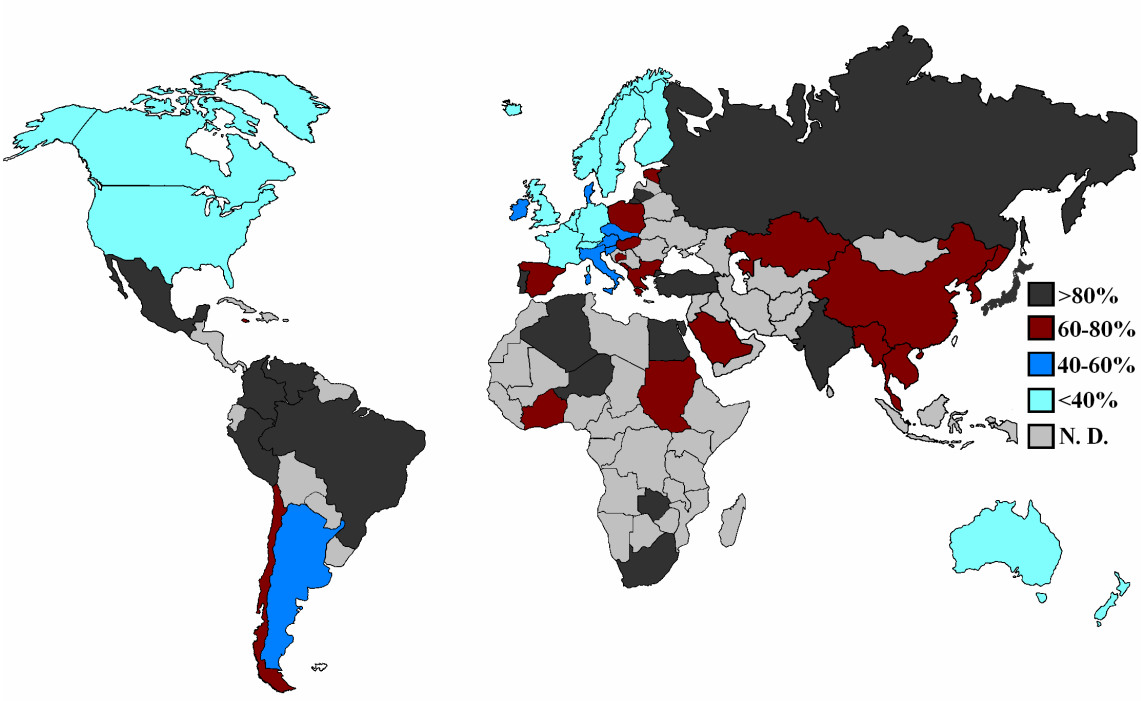


Fig. 1.3. Prevalence of *H. pylori* in human populations worldwide. The map was based on the data collected in Lunet *et al.* (137) and completed with other studies (10, 62, 173, 240). N. D. stands for places where a consistent study about *H. pylori* prevalence has not yet been conducted.

1.4 *H. pylori* possible transmission routes between the human population

Even though the prevalence of *H. pylori* worldwide reaches 50%, the bacterium has not yet been consistently isolated outside the human GI tract. This begs the question: how did *H. pylori* became so widespread among the human population? Numerous epidemiological studies have been conducted to identify the factors influencing the transmission of this pathogen. Socioeconomic status is clearly the most important determinant for the development of *H. pylori* infection, with poorer/lower social classes exhibiting much higher prevalence (158), which is also in accordance with differences found between underdeveloped and developed countries described in the previous section. This factor encompasses conditions such as levels of hygiene, density of living, sanitation

and educational opportunities, which have all been individually identified as markers of the bacterium presence.

Largely based on epidemiological and microbiological evidence, several routes of transmission have been conjectured (Fig. 1.4). Many authors agree that the relative importance of these routes in the transmission of the bacteria is likely to vary between developing and developed countries (154, 192). The most relevant in overall terms are now addressed in detail.

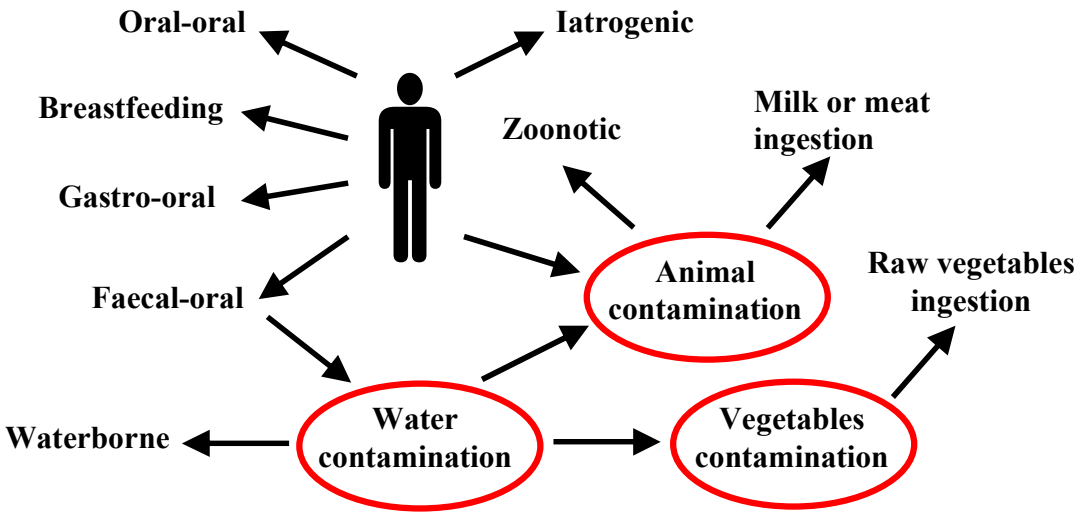


Fig. 1.4. Suggested transmission route pathways. The red circles identify possible reservoirs outside the human host.

1.4.1 Person-to-person transmission

Person-to-person transmission is widely seen as the most probable route of infection, mainly because of the apparent failure to consistently isolate *H. pylori* in places other than the human GI tract. Furthermore, numerous epidemiological studies have consistently identified domestic overcrowding and infection of family members as a risk factor for *H. pylori* transmission (e.g. 130, 202, 208). Roma-Giannikou *et al.* (207) found a strong homology of the *H. pylori* genome in infected members of the same family, and clustering of *H. pylori* infection in families has been widely reported in other studies (e.g. 140).

Although the authors suggest that this evidence supports the hypothesis of person-to-person transmission, exposure of a family to an alternative common source still remains a possibility.

The most relevant pathways of person-to-person transmission encompass the gastro-oral, oral-oral and faecal-oral routes. Breastfeeding and iatrogenic transmission are also included as alternative ways for the dissemination of the pathogen.

The gastro-oral transmission has been postulated mainly for young children, among whom vomiting and gastro-oesophageal reflux are common. In a study by Parsonnet *et al.* (187), vomitus from infected subjects and surrounding air were sampled for *H pylori*. All vomitus samples were positive (often recovering the bacterium in high quantities), and even the surrounded air tested positive for 37.5% of the cases. It has also been suggested that exposure to microscopic droplets of gastric juice during endoscope manipulation could explain an higher prevalence of infection in gastrointestinal endoscopists (89).

The oral cavity has been considered to be a suitable reservoir for *H. pylori* subsistence, and identical strains of the pathogen have been detected by polymerase chain reaction (PCR) in the mouth and stomach of infected individuals (117). Oral-oral transmission has therefore been suggested to occur with kissing or, as it happens in some ethnic backgrounds, from mothers to their babies as they pre-masticate their food. However, cultivation of the microorganism from the oral cavity has been sporadic and related to transitory regurgitations of the microorganism from the stomach into the mouth (134). Luman *et al.* attempted to cultivate the microorganism from saliva and the dental plaque of 120 dyspeptic patients with *H. pylori*-associated gastritis but failed to obtain any positive result, despite the fact that 47% of the patients were found to be *H. pylori*-positive on culture from antral biopsies (135). The same authors compared the genotypes of *H. pylori* isolated from patients and their spouses by polymerase chain reaction-restriction fragment length polymorphism and found very little similarity (136). It is however possible that several mechanisms, such as point mutations and intragenic recombination, could enhance strain diversity once the infection is acquired. As society in the more developed countries is becoming more liberal in terms of human relationships, the act of kissing has developed into a more natural practice. This would supposedly imply an

increase of *H. pylori* prevalence and contradicts the downward trend observed in the more developed countries.

It has been suggested that the faecal-oral route for *H. pylori* transmission is very unlikely due to the contact with human bile, to which it is very sensitive, during the passage through the intestine (82, 159). However, the fact that *H. pylori* is able to colonize the duodenum (upper part of the small intestine) and even cause duodenal ulcers, appears to be an inconsistency, and has raised some questions about the exact effect of the passage of the microorganism through the intestine (81). Well-established detection methods based on PCR or enzyme-linked immunoassays systematically identify the presence of the microorganism (e.g. 106, 165, 172), but growth of the bacterium using culture methods has been more elusive, and achieved most of the times from individuals with accelerated gut transit time (134).

The detection by PCR of *H. pylori* in breast milk has also raised the possibility of breastfeeding as a route of transmission (119), even though earlier studies stated that infants born from *H. pylori*-positive women are not more likely to acquire the infection (16). The contamination of milk could be possible if the bacterium survived in the nipples or fingers.

Iatrogenic transmission is another possible transmission pathway. In 1995, a study showed strong evidence that linked contamination of endoscopes and biopsy forceps to *H. pylori* transmission (244). Since then, it is thought that adequate disinfection procedures have been established and that this pathway of transmission is nearly neglectable.

1.4.2 Contact with animals

Including contact with animals as a possible transmission mode is an obvious reasoning, as zoonotic transmission represents one of the leading causes of illness and death from infectious disease worldwide. In the case of *H. pylori* the considered animal vectors include cows (65), sheep (55), cockroaches (100), houseflies (182) and domestic animals (19).

In the first two cases, the suspected route of transmission is mainly by the ingestion of contaminated raw milk. The milk could become contaminated when the breast of a cow or

sheep is in contact with faeces in the soil. Epidemiologic data has shown higher prevalence in shepherds and their families than in the general population (54, 185). The survival and detection of the bacterium in milk is described ahead (see Section 1.4.4).

Inamura *et al.* (100) suggested that cockroaches, which usually live in unsanitary environments, may contaminate foods and food containment areas such as pantries. The authors studied the survival of *H. pylori* on the external surfaces (legs and body) and excreta of *H. pylori*-exposed cockroaches and found that the microorganism was culturable from the excreta of the exposed group for 24 h postchallenge, but not from the external surfaces. A similar study was also performed with houseflies (77). In this case, *H. pylori* was recovered from external surfaces for up to 12 h and from gut and excreta for as long as 30 h postchallenge. The negative detection after 30h was attributed to the appearance of other Gram-negative bacteria that overgrew the cultures. However, when this study was repeated exposing the houseflies to *H. pylori*-contaminated human faeces instead of *H. pylori* grown on agar plates, the microorganism was not cultured from any of the locations (182).

Epidemiological studies showed controversial results in respect to the risk of the presence of domestic animals in the household (e.g. 17, 108, 132). *H. pylori* has not been found in dogs and only very rarely in cats' stomachs (59, 166), and it has been suggested that the presence in animals is of human origin (38, 59). Recent work has identified *H. pylori* by PCR in the bile of cats, thus increasing the chance of this animal as a vector (19).

It is now known that nearly each animal is colonized by its own endogenous *Helicobacter* spp. Like *H. pylori*, that has co-evolved with humans to be highly specialized in the colonization of the human stomach, these bacteria have specialized in colonizing the GI tract of their specific natural host. Presumably, therefore, *H. pylori* will find fierce competition by these other *Helicobacter* spp. in the search for essential nutrients.

1.4.3 Water ingestion

A great number of epidemiological studies have investigated drinking water, or drinking water-related conditions, as a risk factor for *H. pylori* infection. Although Yamashita *et al.* reported the absence of an association between prevalence of *H. pylori* and the type of water supply in Japan (267) and Ilboudo *et al.* found that in Burkina Faso prevalence was also not associated with the presence or absence of running water in homes (98), the majority of the other studies support a more or less evident correlation between these parameters. For instance, Klein *et al.* (120) reported that children from high-income families in Peru whose homes were supplied with municipal water were 12 times more likely to be infected than those from the same socio-economic status whose water supply came from community wells. Results implicating water were also obtained in studies performed in Germany (88), Kazakhstan (173), Brazil (138), Mexico (45), Argentina (178) and Japan (107).

Despite all these data supporting water as an infection vehicle, the low culturability time of the bacterium when exposed to water in the planktonic state undermines this possibility. Two hypothesis have therefore been advanced to still sustain water as a credible transmission route: either the time of survival will increase if the bacteria is embedded in an EPS/microorganism matrix – biofilms, or the techniques used to recover the bacteria do not detect bacteria still capable of causing infection – that can loosely be termed as viable but non-culturable bacteria (VBNC). Because these hypotheses are the framework where the experimental part of this thesis relies on, evidence supporting or rejecting these possibilities will be referred in more detail in the next Chapters.

1.4.4 Food ingestion

At least two epidemiological studies have found a positive relationship between the consumption of uncooked vegetables and *H. pylori* transmission (74, 94). Raw vegetables are suspected to be vulnerable to *H. pylori* colonization when contaminated water is used for washing or irrigation. It is important to bear in mind that this route assumes that *H. pylori* is also able to survive in water and has therefore all the problems associated with this possible transmission route. No reports have been found about cultivation methods or

molecular biology procedures trying to detect the microorganism from these products. Survival studies indicate that inoculated *H. pylori* (temperature: 8 °C; inoculation density $\approx 10^6$–10^7 CFU/g) dropped below detection limits at 4 days in sanitised lettuce and carrot samples, and at 5 days in sterilised carrot (72). In a different study, survival of the microorganism lasted for up to 2 days in leaf lettuce (4 °C; $\approx 10^2$ CFU/g) (195).

Milk is another type of food implicated as a possible transmission vehicle by epidemiological studies. Constanza *et al.* correlated infection with the intake of milk products in Mexico (45). Conversely, an epidemiological study in Italy reported an inverse correlation between the elevated consumption of milk and *H. pylori* prevalence (211). The differences obtained in both studies might reflect variable milk microbiological quality between these two countries. Interestingly, a recent study in Poland (also referred to in Section 1.4.2) showed that prevalence in shepherds and their families was 20-30% higher than in farmers with no contact to sheep (185). They understandably attributed this difference to the contact with animals (zoonosis), but failed to consider a probably higher quantity ingestion of raw milk by the shepherds and their families as a variable. Previously, in 1999, Dore *et al.* found similar results in a community of Sardinian shepherds and their families (54), but the research group went on to try and detect *H. pylori* presence in sheep milk. They were able to report the recovery of viable *H. pylori* from raw milk samples on two separate occasions (55, 56), but failed to confirm the survival of the microorganism after pasteurization of the milk. Furthermore, a larger screening of 400 raw sheep milk samples performed in Turkey detected no viable *H. pylori* (243). The pathogen has also been cultured from one sample of raw cow's milk in Japan (65), and in the same work PCR demonstrated the presence of the ureA gene of *H. pylori* in 13 of 18 (72.2%) raw milk samples and in 11 of 20 (55%) commercial pasteurized milk samples. Survival studies indicate that the bacterium remains culturable in pasteurized milk for 5 days (4 °C; $\approx 10^4$ CFU/mL) (195).

Poms and Tatini also studied the survival of *H. pylori* in other commercially available food products, such as yoghurt, chicken meat and tofu (195). The bacterium was cultured for 1, 2 and 7 days, respectively (4 °C; $\approx 10^2$ CFU/g). Differences in the culturability could be explained by the work of Jiang and Doyle (104), who, based on the effect of

environmental and substrate factors on survival and growth of *H. pylori*, have stated that the microorganism usually exhibits extended survival in low acid/high moisture environments. Also, autochthonous microbiota present in the yoghurt, such as *Lactobacillus* and *Bifidobacterium*, have been shown to inhibit the survival of *H. pylori* (256, 260).

1.5 Drinking water distribution systems and associated biofilms

Chemically and biologically safe drinking water is one of the main goals that both drinking water companies and governments worldwide try to achieve. In 2002, 1.1 billion people lacked access to improved water sources, which represented 17% of the global population. This assumes particular importance because diseases associated with unsafe water, sanitation and hygiene cause approximately 1.7 million deaths each year according to the World Health Organization (201). Diseases that mostly contribute to this toll are diarrhea and malaria which occur mainly in Africa. Although in the developed countries a waterborne disease is seldom lethal, there have been numerous outbreaks causing health concerns (e.g. 13, 91, 125, 215). Outbreaks are usually associated with malfunctioning of the drinking water treatment plants, which fail in maintaining a minimal disinfectant concentration in the water essential to prevent the growth/survival of pathogenic organisms present in the drinking water distribution system. Outbreaks in the last few years have been associated with different types of microorganisms, such as bacteria (*E. coli* O157, *Salmonella* spp, *Campylobacter* spp, *Vibrio cholerae* and *Shigella*), protozoa (*Cryptosporidium* and *Giardia*), intestinal helminths (*Ascaris lumbricoides*, *Trichuris trichiura* and hookworms) and even virus (norovirus). Water supplies can become contaminated when fecal discharges from infected humans, animal pets, farm animals, and wildlife are released to the water environment (69).

Biofilms can be defined as communities of microorganisms attached to a surface and encased within an extracellular polymeric substance (EPS) matrix. They are heterogeneous in structure and include characteristics such as cell clusters (or frond

formation), grazing eukaryotic predators, and channels that are formed if the fluid surrounding the biofilm is in movement (Fig. 1.5). Because bacteria will attach to virtually any surface, these structures are nearly ubiquitous and its presence has been thoroughly studied in such diverse areas as in prosthetic devices or pipe surfaces. It is now widely accepted that the main reason behind the formation of biofilms is the better chance of survival of bacteria in the microenvironments generated by the heterogeneous structure and activity of the complex consortia (111).Their resilience has been related to altered physiology (induced by intercellular chemical signaling, quorum sensing or the microenvironment itself) and protection by the EPS matrix that it is produced. Therefore, typical biocidal strategies will only be partially effective against this type of complex (224). This assumes particular importance because several pathogenic species that are found in the environment can form or incorporate into these complex structures (80).

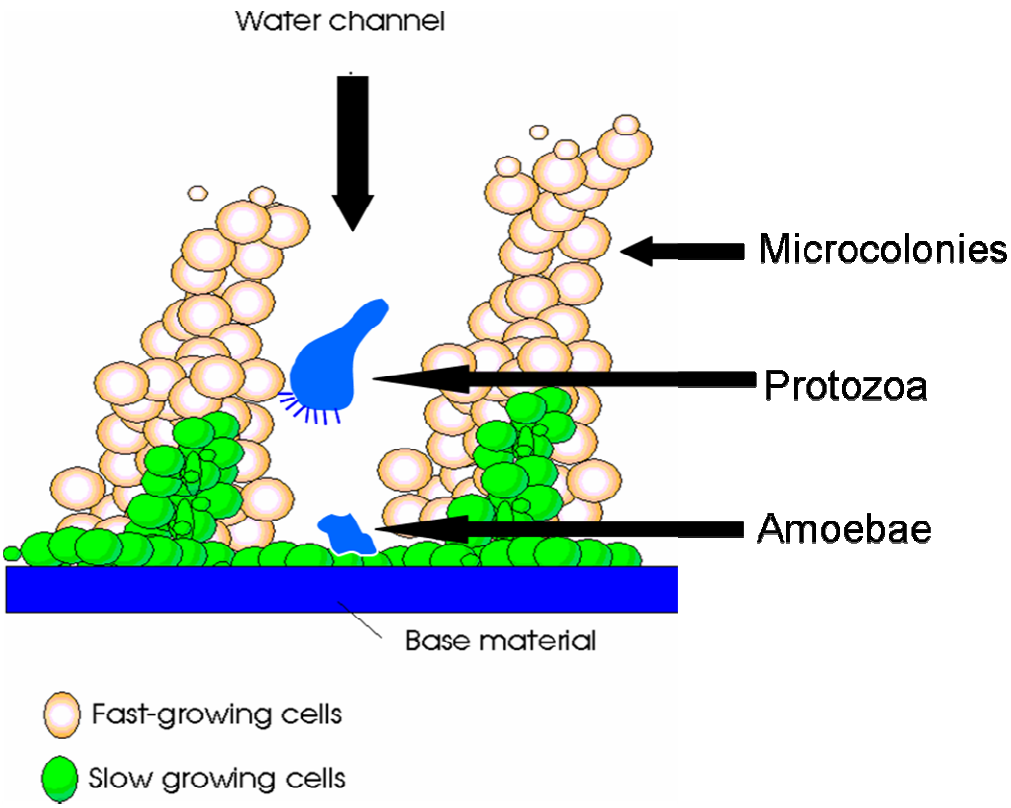


Fig. 1.5. Open architecture structure of biofilm with fronds, water channels and grazing eukaryotic predators. Reproduced from Keevil *et al.* (113).

After the recognition of biofilms as an important area of study within microbiology and other fields of research (47), several biofilm formation systems were developed. The main concern of the designers was to mimic the in situ situations and also obtain reproducible results. The modified Robbins device and the flow cell are arguably the best recognized in the area of drinking water biofilms (23, 118), but chemostats have more recently arisen as a way to more easily control environmental conditions, obtain reproducible results and reduce the possibility of leaks that can be hazardous to the operator when the system is spiked with pathogens (109). Static adhesion is also currently being used as high throughoutput method. As the name itself indicates, static adhesion is meant to include all assays in which a microbial suspension remains stationary with respect to an exposed substratum surface.

2 *Helicobacter pylori* detection in heterotrophic biofilms

As it was mentioned in Chapter 1, biofilms can provide a shelter that would support the existence of viable *H. pylori* for longer in drinking water systems. With the work described in this Chapter it was intended to assess the ability of the bacterium to attach and integrate in a heterotrophic biofilm formed of endogenous species of drinking water systems. For that, a biofilm formation device, named two-stage chemostat system was re-developed to safely work with the pathogen. Tracking of *H. pylori* amid other species was possible after the development and testing of a species-specific peptide nucleic acid (PNA) probe. The probe was firstly applied to biofilms grown on a laboratory biofilm formation device, and afterwards to sections of pipes obtained from real drinking water systems.

2.1 Introduction

By 2002, two research groups had already reported the *in situ* detection of *Helicobacter* spp. in biofilms associated with drinking water systems, using a nested PCR technique (30, 186). The same technique had previously allowed Mackay *et al.* to demonstrate that *H. pylori* could persist for up to 192 h in a mixed species drinking water biofilm after inoculation in a modified Robbins device (141). What any of these experiments failed to provide, however, was spatial information about *H. pylori* presence: would the pathogen preferably attach directly to the surface or would it incorporate into stacks or fronds and, if so, would it favor any of the microenvironments present in such a complex structure? Attaining this knowledge is essential in order to understand possible structural/functional relationships between different microorganisms and *H. pylori* when the pathogen is found in biofilms from drinking water systems.

2.1.1 *In situ* hybridization and microscopy techniques

In situ hybridization (ISH) is one of the techniques available to identify the bacteria that compose heterotrophic microenvironments and determine where they are predominantly located. It is based on the ability of RNA to form duplex strands by providing a RNA complementary sequence (probe) to one already existent in the cell (target), usually in the form of rRNA, and providing the necessary conditions for them to hybridize. A reporter molecule - fluorescent (FISH) or chromogenic (CISH) are the more common - can be attached to the probe so that it is possible to be tracked by fluorescence or brightfield microscopy. The FISH method is however a more widely established technique which has been used in diagnostic and other fields of microbiology. It is considered to be a reliable way to detect and visualize microbial species, and has been successfully adapted for the study of bacteria in biofilms in several reports (e.g. 103, 144, 171, 176). Besides providing spatial information, coupling of the FISH technique with microautoradiography has been used to provide an indication of the physiological state of microbial cells in a consortium of microorganisms (169, 236).

The use of a fluorescence microscope has been in the meantime substituted by the more efficient epifluorescence microscope. Epifluorescence is an optical set-up for a

fluorescence microscope in which the objective lens is used both to focus ultraviolet light on the specimen and collect fluorescent light from the specimen. It allows fluorescence microscopy to be combined with another type of microscopy on the same microscope such as the episcopic differential interference contrast (EDIC) microscopy. EDIC has provided a rapid, real time analysis of biofilms on opaque, curved, natural or man-made surfaces without the need for cover slips and oil (112). EDIC, coupled with epifluorescence microscopy (EDIC/EF), has been successfully used to visualize the 3-D biofilm structure (254), iron biomineralization (26), and the location of specific pathogens such as *Legionella pneumophila* (205) or *Campylobacter jejuni* (32) within the biofilm.

2.1.2 Peptide nucleic acid hybridization probes

In the early 1990s, Nielsen *et al.* (168) reported the development of a synthetic DNA analogue, named peptide nucleic acid (PNA) (Fig. 2.1). This molecule proved to be capable of forming PNA/DNA and PNA/RNA hybrids of complementary nucleic acid sequences, and its neutrally charged polyamide backbone made FISH procedures easier and more efficient for different reasons:

- Hybridization could be performed efficiently under low salt concentrations (181), a condition that promotes the destabilization of rRNA secondary structures and results in an improved access to target sequences that would be elusive using conventional FISH.
- Hybridization does not suffer from the electrostatic repulsions that occur when a DNA oligonucleotide is used. Because of the lower free energy of the hybrid a better specificity and more rapid hybridization kinetics of the probe is achieved (193).
- Diffusion through the cell membrane and naturally occurring microstructures such as the EPS biofilm matrix might be easier even in Gram-positive bacteria due to the hydrophobic character of PNA as compared to DNA (57).
- It is not a substrate for the attack of proteases or endonucleases (53).

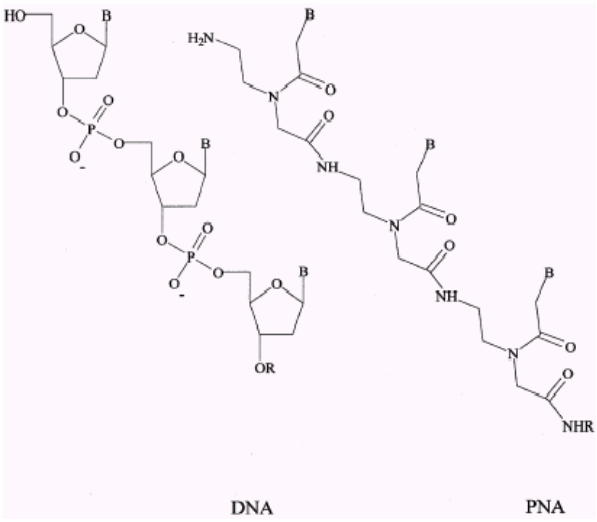


Fig. 2.1. Comparison between the DNA and PNA chemical structure. From Stender *et al.* (231).

Taking advantage from these features, Perry-O'Keefe *et al.* (193) developed a new procedure for the simultaneous detection of both Gram-negative and Gram-positive species, something never accomplished using DNA probes. A more extensive review of PNA applications in FISH technology can be found in Stender *et al.* (126, 231).

Apart from interactions within multicellular organisms, biofilms are probably the naturally occurring microenvironment where the behaviour of unicellular organisms is more difficult to understand and predict, due to the cell to cell signaling and other chemical interactions. An assessment *in situ* of the type of microorganisms present in biofilms seems to be the next logical step in the comprehension of these complex microstructures and the recent application of PNA probes might open new horizons if the simultaneous determination of different species in planktonic bacteria can be transposed to these systems.

2.1.3 Software available for probes evaluation

There is now a large array of programs that allow the evaluation of newly designed probes online. The most recognized is the Basic Local Alignment Search Tool (BLAST) (152), where several types of sequence analysis can be performed. To check for the specificity of a small sequence, a special subprogram was designed – the "Search for short

nearly exact matches". Although this site performs efficient analysis in a very short period of time, the fact that it uses a very large database containing both DNA and RNA (Genbank-nr) and software that is not specific for probe searches makes it more difficult to handle. A new generation of programs that specifically address these issues have therefore been created, namely the Ribosomal Database Project (RDP-II) (40), the European ribosomal RNA database (266) and the probeBase (133). These new sites use rRNA libraries only and have developed user-friendly software, features that decrease the time needed for the design and evaluation of a new probe.

2.2 Material and methods

2.2.1 Culture revival and maintenance

Helicobacter pylori NCTC 11637 was obtained from the PHLS (Colindale, UK) as freeze-dried ampoules, and rehydrated for 3 minutes in Brucella broth (Oxoid, Basingstoke, UK). The suspension was subsequently used to inoculate several plates of Columbia Agar (Oxoid) supplemented with 7% (v/v) of defribinated horse blood (Oxoid), hence designated Columbia Blood Agar (CBA). Plates were incubated at 37^{o}C under microaerophilic conditions using Campygen gas packs (Oxoid) in a closed container and subcultured every two or three days.

2.2.2 Two-stage chemostat model system

As mentioned in Section 1.5, the chemostat system has been developed not only to simulate conditions found in a water system, but also for providing safety handling of pathogens within biofilms. In this study, two 2L glass vessels (Jencons, Leighton Buzzard, U.K.), where the first acted like a seed vessel and the second provided typical environmental conditions for biofilm growth, were linked in series (Fig. 2.2). A top-plate, manufactured by Brighton Systems Ltd. (Newhaven, U.K.) in accordance to the design described in Keevil *et al.* (110), sealed the top of the vessels. The top plates were titanium-made in order to eliminate extraneous iron from the culture and have been designed to allow the insertion of several devices to control/monitor the behavior of each

vessel. Both vessels were therefore equipped with a weir device, to control the volume of the water inside the vessels; an anti-grow back device, that would feed the vessels while preventing microorganisms from crawling back into the feeding area; a temperature sensor, connected to a proportional integral derivative unit system (Anglicon BS910, Brighton Systems Ltd.) that in turn insured a constant temperature of the water inside the vessels by means of an external electrical pad; a sample port hood (Brighton Systems Ltd.), which was also titanium-made, to allow regular sampling of the water without the need to remove the top-plate, and also a filter vent (Fisher Scientific UK Ltd., Loughborough, U.K.), to avoid pressure build-ups inside the vessel. The unused holes were closed with silicone rubber bungs (Merck & Co. Inc, Whitehouse Station, U.S.A.). After the insertion of all devices, the vessels were autoclaved at 121 °C for 15 min. Both vessels were then placed on top of magnetic stirrers (500 series II, LH Engineering, Stoke Poges, U.K.), and filled with 1L of water. The stirrer speed was set to 300 rpm to ensure liquid homogenization as well as sufficient oxygen concentration in the water. The seed vessel was connected to a 20L autoclavable propylene carboy (Fisher Scientific UK Ltd.), which would pump 0.05 L/h of water through a multi-channel peristaltic pump (Minipuls 2, Gilson Inc., Middleton, U.S.A.) to produce a dilution rate of 0.05 h^{-1}. To ensure reproducibility, the conditions in this vessel remained constant. A constant 0.05 L/h flow from this vessel was supplied into the biofilm-growing vessel, using the same pump. Additional water was added to this vessel from a second propylene carboy connected to a MHRE22 peristaltic pump (Watson-Marlow, Falmouth-Cornwall, U.K.) to produce an overall dilution rate of 0.2 h^{-1}. Using the weir system connected to an 802S peristaltic pump (Watson-Marlow), effluent from the second vessel was collected to a waste container. The temperature in both vessels was set to 30 °C. All the water used in this study was tap-water that was filter-sterilized using 0.2 μm pore size nylon membranes (Fisher Scientific UK Ltd.), unless otherwise stated.

Fig. 2.2. Experimental setup of the two-stage chemostat system. The seed vessel (left) feeds the biofilm-growing vessel (right) by means of a peristaltic pump. The electrical device seen on the far right is the proportional integral derivative unit system used to control the temperatures.

2.2.3 Coupon preparation

At the School of Biological Sciences of the University of Southampton workshops, rectangular coupons of stainless steel 316 (RS Components Ltd., Corby, U.K.) were prepared with an area of 1.2 cm^2 (1.2×1cm), and a 1 mm diameter hole was drilled in one of the ends. This hole allowed coupons to be suspended from titanium wire TI005125 of 0.5mm diameter (Goodfellow Cambridge Ltd., Huntingdon, U.K.), and assembled in the form of three coupons per set. Before the sets were inserted in the second vessel they were immersed in acetone (Bibby Sterilin Ltd., Stone, U.K.) for 5 min. with the purpose of removing any organic carbon compounds adsorbed to them (Fig. 2.3), and then autoclaved.

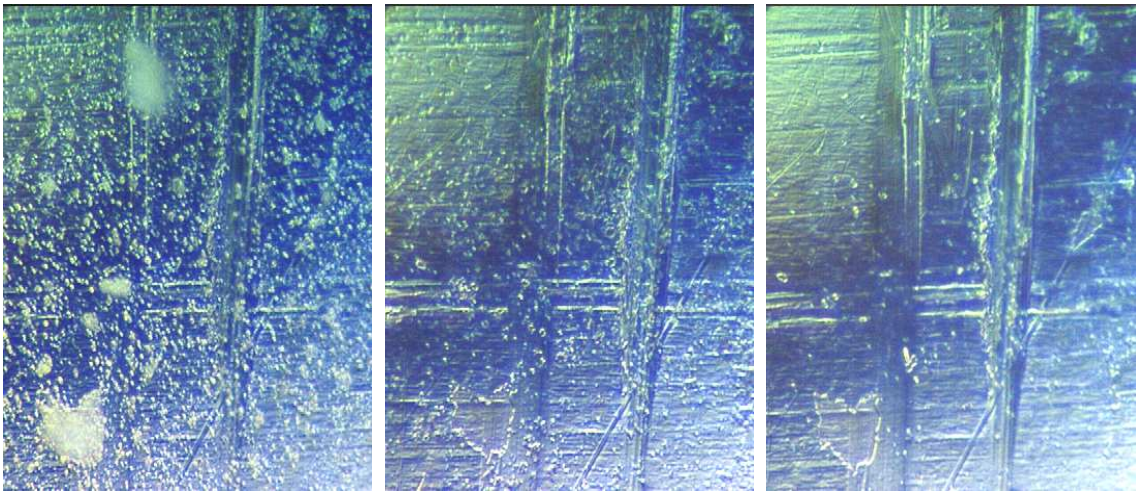


Fig. 2.3. EDIC/EF microscopy visualization of a section of a coupon where biofilm was grown before (left), and the same section after cleaning with acetone for 2 (center), and 5 minutes (right). Mag. ×1000.

2.2.4 Monitoring of sessile and planktonic bacteria in the chemostat system

The heterotrophic consortium of microorganisms collected during the first filtrations of the tap water was resuspended in 10 mL of a phosphate buffered saline solution (Oxoid). Of these, 5 mL were used to inoculate the seed vessel, while the remaining solution was stored at 4 °C in a cold room. Regular sampling was performed in both fermenters to enumerate planktonic bacteria, measured as colony forming units (CFU) grown on R2A (Oxoid) and CBA agar. After 10 days, sets of sterile coupons were introduced in the second vessel for biofilm growth. Coupons were either directly used for microscopy observations or scraped for heterotrophic plate counts (HPC) on R2A and CBA agar. These analyses were performed in a first experiment that took approx. 70 days and where no *H. pylori* was inoculated.

Before microscopy observations, coupons were either left unstained, or stained with acridine orange (AO), which binds to RNA and DNA, and cyanoditolyl tetrazolium chloride (CTC) that detects respiratory activity (151). For all procedures, coupons were removed from the chemostats, rinsed twice in sterile distilled water and allowed to air-dry for 10 min. If staining was intended, a solution of 0.3 g/l of AO hemi zinc salt (Sigma-

Aldrich Company Ltd., Gillingham, U.K.) was then directly applied over the coupon for 2 min at room temperature. Alternatively, coupons were covered with 5 mM of CTC (Park Scientific Ltd., Northampton, United Kingdom) for 1 h at 30 °C. Coupons were then washed with sterile distilled water and allowed to air-dry for another 10 min, before visualization under the microscope. The staining procedures were all performed in the dark.

2.2.5 Inoculation of the chemostat system with *H. pylori*

In a second experiment and after re-sterilization of the whole system, the remaining 5mL of the heterotrophic consortium of microorganisms collected during the first filtrations of the tap water were inoculated in the seed vessel. All the remaining parameters of the two-stage chemostat system (flow rates, temperature and stirrer speed) were kept constant, but sets of coupons were introduced immediately after the inoculation of the heterotrophic consortium. After allowing the growth of biofilms for 10 days, the model system was inoculated with *H. pylori* at 10^6 CFU/ml taken from a 2 day-old CBA plate. A set of coupons from the vessel was taken just before inoculation (control) and at different times up to 5 days after inoculation, and the pathogen was tracked using PNA FISH.

2.2.6 Design of PNA oligonucleotide probes

Although several references exist in the literature mentioning DNA probes for *H. pylori*, no probe had been developed using PNA FISH technology. Therefore, several sequences for *H. pylori* NCTC 11637 16S rRNA described in the literature were evaluated (210, 237, 241) for their suitability for this technology. The main adaptation performed on an average DNA oligonucleotide probe is the shortening of the sequence to comply with the optimum of between 12 and 18 bases necessary to achieve an optimal combination of specificity and binding strength in PNA hybridization. For the purpose of checking the impact of this modification, both an advanced BLAST search and a 16S rRNA sequence match analysis at the RDP-II site were carried out. Based on the primer ACT-1 described in Thoreson *et al.* (237), the sequence 5'-(TAATCAGCACTCTAGCAA)-3' was found to fulfill the necessary requirements. The advantage of this sequence is that there are at least two base mismatches for every other microbial species, which will theoretically minimize the

chances of non-specific binding of the PNA probe to the very diverse microflora in the biofilm. However, the option to choose for this more specific sequence meant that a few *H. pylori* strains would also not hybridize with the probe (Table 2.1). A fluorescent carboxyfluorescein (FAM) molecule was attached to the 5' terminal of the probe. When attached to DNA or PNA probes, this molecule has an absorption maximum of ~490 nm and an emission maximum of ~510 nm (which in pratical terms means that it fluoresces mostly green). It was originally chosen because a double-staining procedure with dyes that predominantly fluoresce in the red channel, such as CTC or AO, was intended.

Table 2.1. Alignment of partial 16S rRNA of *H. pylori* and related microorganisms sequences. Base differences between the target sequence and other sequences are in bold and underlined.

16S rRNA sequence (5'-3')	Species	Strain	Mismatches
U U G C U A G A G U G C U G A U U A	*H. pylori*	NCTC 11637	0
U U G C U A G A G U G C U G A U U A	*H. pylori*	26695	0
U U G C U A G A **A** U G C U G A U U A	*H. pylori*	U00679	1
U U G C U A G A G **A** U G - - G A U U A	*H. cinadei*	ATCC 35683	3
U U G C U A G A G **A** U G - - G A U U A	*H. fennelliae*	NCTC 11612	3

2.2.7 PNA probe preparation and hybridization procedure

The PNA probe (Oswel, Southampton, UK) was supplied as a freeze-dried solid. The probe was dissolved in an aqueous solution of 0.1% of trifluoroacetic acid (Sigma-Aldrich), with a final concentration of 200 μM, and stored at -20 °C in the dark. This solution was then used to create a hybridization solution of 200 nM (Table 2.2), which was also stored in the dark but at - 4 °C.

Table 2.2. Concentrations of chemical compounds and function in the hybridization solution. All chemicals were obtained from Sigma-Aldrich.

Compound	Concentration	Function
Dextran Sulfate	10% (w/v)	Accelerates the rate of nucleic acid hybridization by decreasing the volume of solvent available to the probe
Sodium Chloride	10 mM	High salt concentration, or ionic strength, also increases the reaction rate. However, it also stabilizes secondary structures of rRNA and mismatched hybrids.
Formamide	30% (v/v)	As an organic solvent, it reduces the thermal stability of double-stranded polynucleotides, so that hybridization can be performed at lower temperatures.
Sodium Pyrophosphate	0.1% (w/v)	Part of the Denhardt's solution, it reduces non-specific binding of probe to nitrocellulose membranes.
Polyvinylpirrolidone	0.2% (w/v)	Also part of the Denhardt's solution.
FICOLL	0.2% (w/v)	Also part of the Denhardt's solution.
Disodium EDTA	5 mM	Removes free divalent cations that strongly stabilize PNA-RNA duplexes, by acting as a chelator.
Triton X-100	0.1% (v/v)	As a detergent, it affects membrane permeabilization.
Tris HCl (pH 7.5)	50 mM	Acts as a buffer to control the pH of the hybridization, as variations in pH significantly affect hybridization temperature
PNA probe	200 nM	Hybridizes with the target 16S rRNA sequence and can afterwards be detected by EF microscopy.

The hybridization procedure was done according to Stender *et al.* (251) with slight modifications to ensure an improved detection. All chemicals were obtained from Sigma-Aldrich. Prior to hybridization, stainless steel coupons with biofilm attached or glass

slides with smears of *H. pylori* or other bacteria were immersed in 90% (v/v) ethanol for 10 minutes, and left to air-dry. Approximately 20μL of filter-sterilized hybridization solution was then dispensed on the coupons/slides. These were covered with a glass coverslip and incubated for 90 minutes at 63 ^{o}C in a moist atmosphere. Following hybridization, coupons/slides were washed in a pre-warmed solution containing 5 mM Tris Base, 15 mM NaCl and 1% (v/v) of Triton X (pH 10) at 63 ^{o}C for 30 min. The coupon/slide was then allowed to air dry and examination under an EDIC/EF microscope occurred in the following 72 hours. If an EF microscope was used, a drop of mounting oil was added and the coupon covered with a coverslip.

2.2.8 Field studies

Pipe sections containing biofilm were taken from real drinking water distribution systems from Riga (Latvia), Minho (Portugal) and Yorkshire (UK). The material of the pipes was cast iron in Riga and Yorkshire. In Minho the samples were taken from cement, cast iron and high density polyethylene (HDPE) pipes. The pipe sections were transported under refrigeration and a moist atmosphere and, if necessary, small coupons were prepared at the Center for Biological Engineering workshops at the University of Minho. Hybridization and visualization under the microscope were performed in the following two days after arrival to the laboratory. Hybridization of the pipe sections was performed following the protocol described in 2.2.7.

2.2.9 Microscopy observations

Several microscopes were used in this study (Table 2.3). In the University of Southampton, biofilms were either visualized using an EDIC/EF (Best Scientific, Swindon, U.K.) or an EF (Nikon UK Limited, Kingston upon Thames, U.K.) microscope. In University of Minho, two different EF microscopes were used (Leitz, Bielefeld, Germany and Zeiss, Oberkochen, Germany). All microscopes were equipped with a digital camera and possessed at least one filter that was sensitive to the signaling molecule of the PNA probe. Filters that were not able to detect the probe were used as negative controls.

Table 2.3. Details of each of the microscopes used in this study.

Microscope	Location	Channels/Filter sets[a]			
EDIC/EF Best Scientific	Southampton	EDIC[3]	DAPI[2]	TRITC[3]	FITC[1]
EF-1 Nikon	Southampton	Rhodamine[3]	GFP[1]	DAPI[2]	
EF-2 Leitz	Braga	LIVE/DEAD[1]		DAPI[2]	
EF-3 Zeiss	Braga	LIVE/ DEAD[1]	GFP[1]	DAPI[2]	TRITC[3]

a – Level of sensitivity of the filter set to the probe. [1]High, [2]Medium, [3]No sensitivity.

2.3 Results and discussion

2.3.1 HPC of planktonic and biofilm bacteria

In the seed vessel, and after a slight decrease in the bacterial counts after the two stage model was inoculated and operated in a continuous mode, most probably due to the time cells needed to adjust to the new conditions (lag phase) and the dilution factor, the numbers started to increase and appeared to reach a first plateau, between the 9th and 16th day after inoculation, slightly above 10^5 CFU/ml on R2A agar (Fig. 2.4). Similarly, and in the biofilm-growing vessel, a first plateau was reached after the 9th day, after the numbers suffered a sudden increase following the inoculation, due obviously to the feed from the seed vessel. However, HPC increased again after 21 days, reaching a second plateau at around 10^6 CFU/ml in the first vessel, and 7×10^5 CFU/ml in the second one. These results are similar to others already obtained using the biofilm-growing vessel at the same dilution rate – between 10^4 and 10^6 CFU/ml although for 20 and 40 °C (203). The generally lower total numbers of planktonic bacteria in this vessel, compared to the seed vessel, are due to the higher dilution rate at which this vessel is operated (0.2 h^{-1} compared to 0.05h^{-1}).

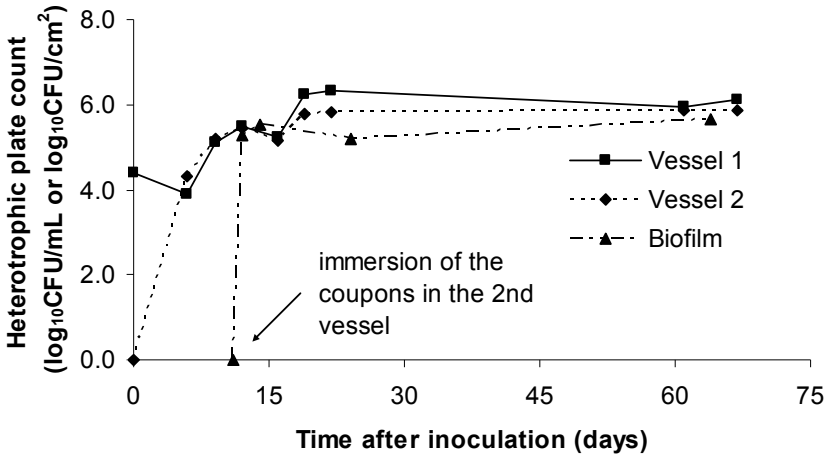


Fig. 2.4. Progress in the HPC obtained in R2A for planktonic bacteria in the two chemostats and in biofilms (n=3).

Even though the second increase in HPC in the biofilm-growing vessel could have been derived from the insertion of the biofilm coupons after day 10, the fact that it could also be observed in the seed vessel appears to indicate that this difference was due to naturally occurring dynamics of the microbial population in the seed vessel. In fact, colonies detected on R2A exhibited very different morphologies, denoting the diverse heterotrophic community of microorganisms that are known to inhabit drinking water distribution systems (189). In this particular case, yellow colonies measuring 0.1-0.6 cm diameter, which mostly appeared after day 21, were the main determinant to the observed increase in HPC. Other types of colonies commonly observed throughout the experiment presented white, pink and occasionally orange pigmentation. Formation of brightly coloured, nondiffusible and nonphotosynthetic pigments has been known to be characteristic of some bacteria found in water supplies (69).

Interestingly, biofilm formation appeared to occur mostly within the first day after insertion of the coupons, with HPC ranging from 2 to 5 $\times 10^5$ CFU/cm^2 until day 72. Rogers *et al.* also reported that, using the same system for the study of biofilm formation and growth of *L. pneumophila*, after 24h surfaces of SS and other materials were heavily colonized (203).This represents a much faster rate of biofilm development then the one

presented in other studies (93, 116, 188), and can be partly explained by the depletion of chlorine from the water due to the high residence times in the chemostats and carboys. However, even in a study where elimination of chlorine was achieved by passing the water through granular activated carbons filter columns the steady state took approx. 6-8 days to be attained (37). Therefore, this event has also to be due to the hydrodynamics of the two-stage chemostat, mainly to the vigorous mixing that is provided in the biofilm-growing vessel and provides liquid homogenization. Most types of the colonies identified in biofilms exhibited striking morphological resemblance to the ones of the planktonic phase, implying that the predominant microorganisms in the planktonic phase could adhere and attach to abiotic substrata.

Colony counts on CBA were mainly performed to assess whether this medium could be used for the recovery of *H. pylori* from the chemostats, once it has been inoculated with the pathogen. As expected, this antibiotic-free medium supported the growth of at least two different types of bacteria, with recoveries that could go up to 10^4 CFU/mL or 10^3 CFU/cm^2 being observed. It was therefore decided not to try the recovery of *H. pylori* using this plating medium. It was not until 2003 that Degnan *et al.* published a plating medium supplemented with antibiotics specifically developed to allow large-scale screening of water samples for *H. pylori* (52).

The two-stage system provided stable conditions for monitoring the prevalence of planktonic and biofilm structures for prolonged periods under controlled environmental conditions, despite the complexity of this type of communities. Biofilm formation was also achieved very rapidly, implying that this system can provide a less time-consuming means to obtain reproducible data for biofilm development and colonization by pathogens such as *H. pylori*.

2.3.2 EDIC and EF microscopy of biofilms and sessile bacteria

Observation of non-stained biofilm coupons using EDIC showed that stacks or fronds were ubiquitous in the surface after only one day of exposure, confirming the results obtained by HPC. Stacks exhibited very different morphologies, either in structure or in size. The design of the EDIC microscope allowed the focussing at the base and top of

these structures to determine their height; typically, and in 1 week-old biofilms, they rose between 30-100 μm from the substratum. Additionally, when coupling EF/EDIC images it was observed that stacks generally exhibited strong autofluorescence in certain, if not all, wavelenghts (Fig. 2.5).

Autofluorescence of biofilms has been only occasionally reported (e.g. 97) and it has been well known that certain autofluorescent chemical compounds, such as polyaromatic hydrocarbons (PAH), can be found in drinking water distribution systems (73). Therefore, the local municipal tap water supply was analyzed for chemical compounds that could cause this anomaly. A concentration of 8 ng/l of fluoroanthrene, a non-carcinogenic PAH was detected. Although within the limits of safe drinking water parameters, it might be possible that this compound is able to attach and concentrate within the exopolymeric substances of the biofilm and therefore be able to cause the autofluorescence observed.

It has been postulated that PAH appears in water due to coal tar linings, widely used for many years as corrosion protection of cast iron and steel water pipes, being degraded by biofilms (143). Little PAH is detected in the water in the absence of disinfectant. However, in the presence of disinfectant, the PAH and coal tar particles are released from the biofilm. After the disinfection process has ceased, the PAH slowly decreases as a result of the reformation of biofilm which then takes some time to develop its protective characteristics. Maier *et al.* (143) concluded that the biofilm then begins to break down the PAH. The consorted metabolic activity of high species diversity biofilms to attack normally refractory molecules may support this conclusion. An alternative hypothesis is that the biofilm EPS merely concentrate PAH and that oxidation of EPS by disinfectants such as chlorine or ozone leads to the release of the bound PAH. Further work with non-oxidising disinfectants will be required to investigate this latter possibility.

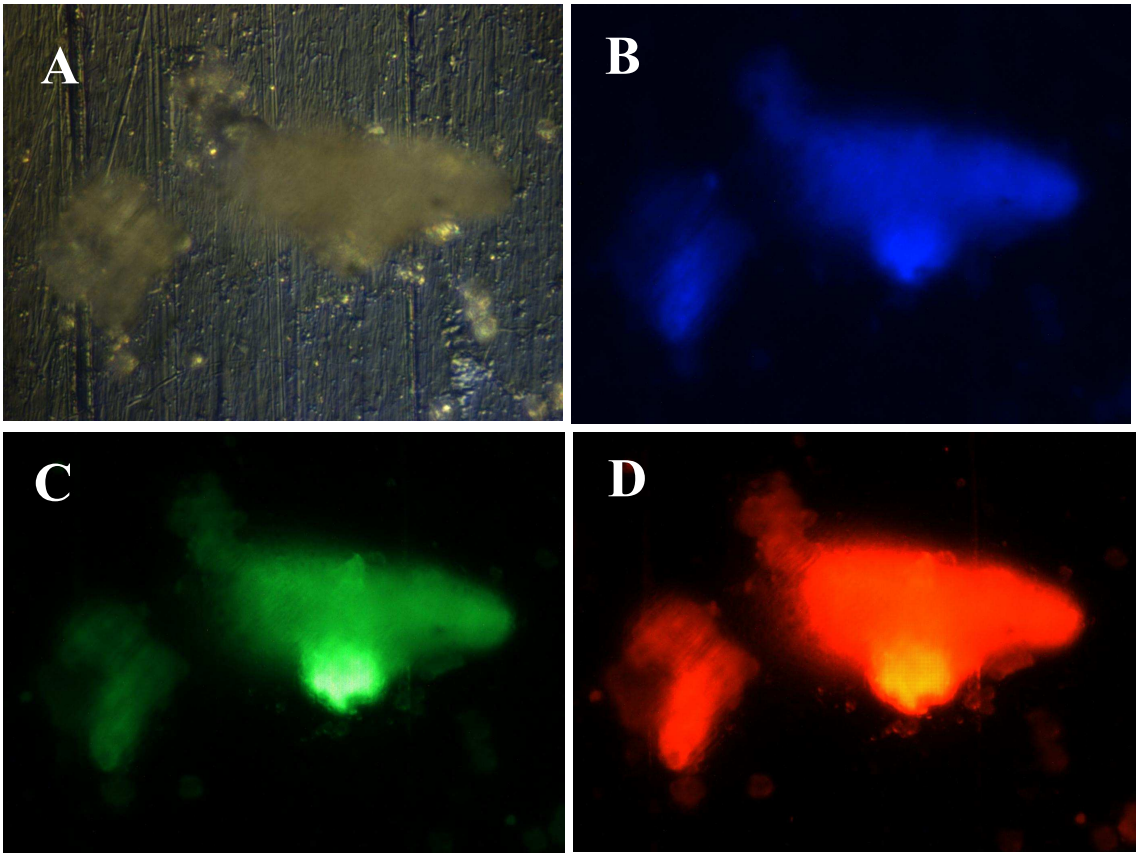


Fig. 2.5. Autofluorescence of a non-stained one week old biofilm stack observed at different channels of the EDIC/EF microscope: EDIC (a); DAPI (b); FITC (c); and TRITC (d). Mag. ×1000.

Besides stacks, certain types of microorganisms attached to the surface were also found to autofluoresce in different filter sets (Fig. 2.6). This implied that a strategy had to be developed so that these microorganisms would not be misidentified as *H. pylori*. The strategy involved analyzing both the morphology of the microorganism and the fluorescence emission in different filter sets. Autofluorescent microorganisms presented very different morphologies, and most of them were easily distinguished from *H. pylori* either because of the cell size, or because of cell shape (Fig. 2.6). Also, it was generally found that autofluorescence could be observed in filter sets that block the fluorescent signal of the labeled probe for *H. pylori*. For instance, for the rods shown in Fig. 2.6, a strong signal was also emitted when viewed through the Rhodamine filter of EF-1, a filter

set that is not able to detect any fluorescence emitted by the *H. pylori* PNA probe. The information from other filter sets was therefore considered to be very valuable for the hybridization experiments.

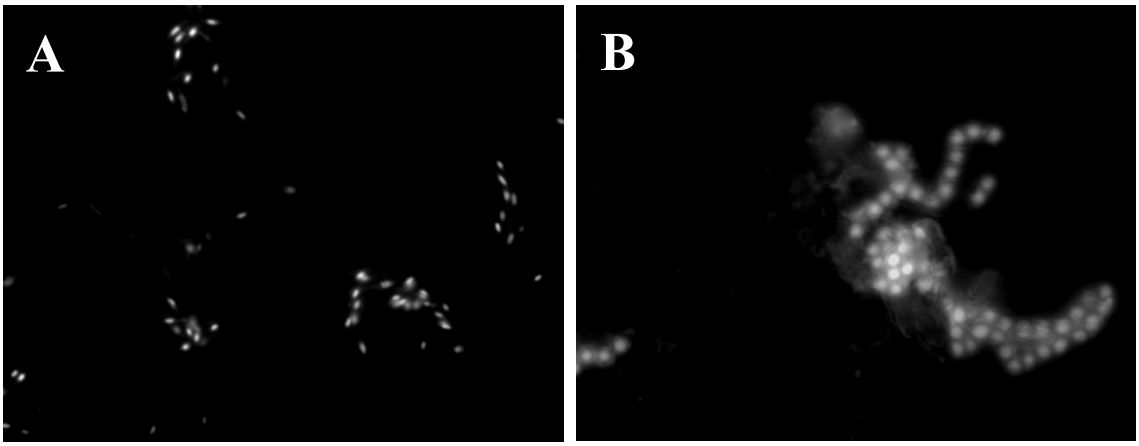


Fig. 2.6. EF-1 images showing autofluorescence of microorganisms attached to the coupons in the GFP channel: Rods 2-4 μm long (a); and a cluster of cocci with approx. 8 μm in diameter each (b).

Also because of autofluorescence, staining with CTC was partly unsuccessful. CTC has been applied to visualize respiring bacteria in different environmental samples (including biofilms), and is indicative of physiological activity within the cell (151). Detection of actively respiring bacteria could only be obtained with a certain degree of confidence when cells were directly attached to the surface, where the autofluorescence effect was less noticeable. AO has been widely use to detect biofilms formed in all ranges of different background (e.g. 128, 129, 184), mostly because it strongly stains microbial nucleic acids and other biological material present in biofilms, such as exopolymers. In this case, AO staining provided a stronger and more widespread fluorescence detection of biofilm, denoting that certain areas of these structures lack the ability to autofluoresce.

The autofluorescence of both microorganisms and stacks could obviously be a drawback for the PNA FISH detection, because of an increase in the risk of *H. pylori* misidentification and the possibility of overlooking the fluorescent signal of the probe against a brighter background. Furthermore, the originally intended dual-staining with

CTC or AO is no longer possible, as the information obtained from filter sets sensitive to those dyes became essential for a correct identification of the pathogen.

Several chemical and physical methods have been tested to quench autofluorescence. Huang *et al.* (97) reported that crystal violet reduced most of the autofluorescent interference on fouling biofilms, but contrast between CTC staining and residual autofluorescence was still poor. Other reagents, such as ammonia-ethanol, sodium borohydride, and Sudan Black B have been tested to reduce autofluorescence on paraffin sections of different types formaldehyde-fixed human tissue with limited success (12). Irradiation with visible and near-UV light has also been claimed to reduce or eliminate autofluorescence of aldehyde-fixed neural tissue (167).

Improved discrimination between the PNA-labeled probe and autofluorescence can also be achieved by labeling the probe with more efficient dyes (presenting more photostability and/or narrower peaks of absorption and emission), such as Alexa Fluor family of dyes (48) or quantum dots (157). That solution would be more efficient if custom designed equipment was provided by filter set manufacturers. Use of other microscopy techniques, such as confocal laser scanning microscopy (127), should also be evaluated for the present case.

2.3.3 PNA probe evaluation

The probe was tested in the laboratory on slide smears of *H. pylori*. Due to the observations described in Section 2.3.2 concerning autofluorescence of non-stained biofilms and microorganisms, *H. pylori* was also checked for autofluorescence. The pathogen showed autofluorescence if analyzed immediately after scraping from the CBA plates, but that autofluorescence was very weak after the hybridization and washing procedure. It was also found that exposure of *H. pylor*i to water would eliminate autofluorescence in less than 1 hour. Strong fluorescence could therefore be attributed to the detection of the PNA-labeled probe. Bacteria cultured from the system using R2A medium were found not to hybridize with the probe. There was also no evidence for non-specific binding of the PNA probe to the general heterotrophic microflora prior to the

introduction of *H. pylori*. Therefore, the probe provided good discrimination between *H. pylori* and other bacteria (Fig. 2.7).

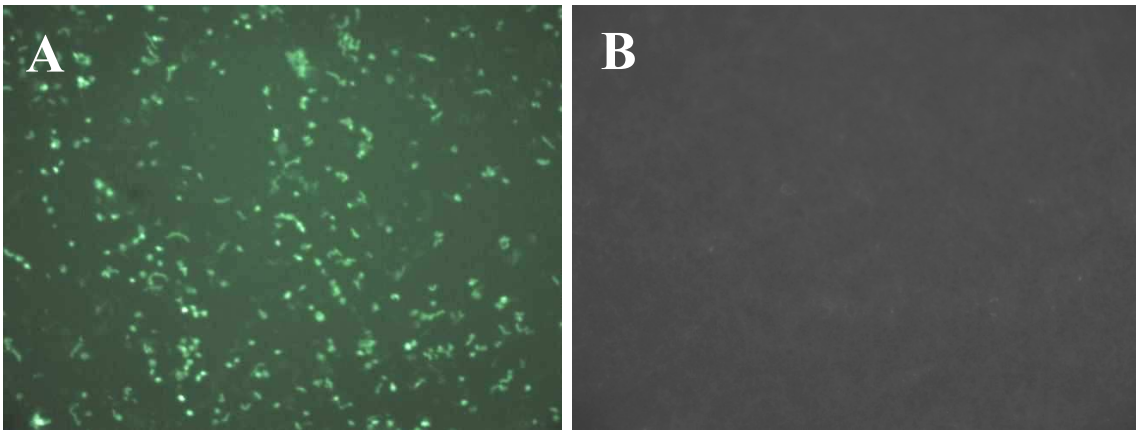


Fig. 2.7. Hybridization of *H. pylori* and other bacterium in slide tests visualized with the EF-1 microscope. Epifluorescence false-color image of hybridized *H. pylori* on a glass slide (a). Epifluorescence image of other bacteria submitted to the same process (b).

Both microscopes in Southampton could detect the hybridized bacteria, but the EF-1 microscope appeared to be more efficient, which can be explained by the absence of oil immersion lenses in the EDIC/EF microscope by the time this study was performed. The EDIC/EF microscope has in the meantime be equipped with an oil immersion lens, and PNA work is now routinely being analyzed with it. The probe appears to emit mainly in the green filter sets of both microscopes, although some fluorescence could also be detected with the blue filters (see Table 2.2 in Section 2.2.9).

2.3.4 Detection of *H. pylori* in heterotrophic biofilms using PNA FISH

In the control coupon observed before the spiking of *H. pylori* in the system and without the PNA probe, autofluorescence of stacks and microorganisms, as described in Section 2.3.2, was also observed. This autofluorescence could also be identified after the inoculation of *H. pylori* and made detection of the pathogen more difficult. Nevertheless, and using the EDIC/EF microscope, *H. pylori* could still be easily detected in the basal layer of the biofilm, and adjacent to the stacks, where the effects of the autofluorescence could not be detected. The EF-1 microscope could even, in some situations, detect some

bacteria incorporated in the EPS matrix of the biofilm (Fig. 2.8). A cell was considered to be *H. pylori* if it exhibited typical morphology and if the fluorescence emission was intense in the probe-sensitive filter sets and faint or non-existent in all other filters.

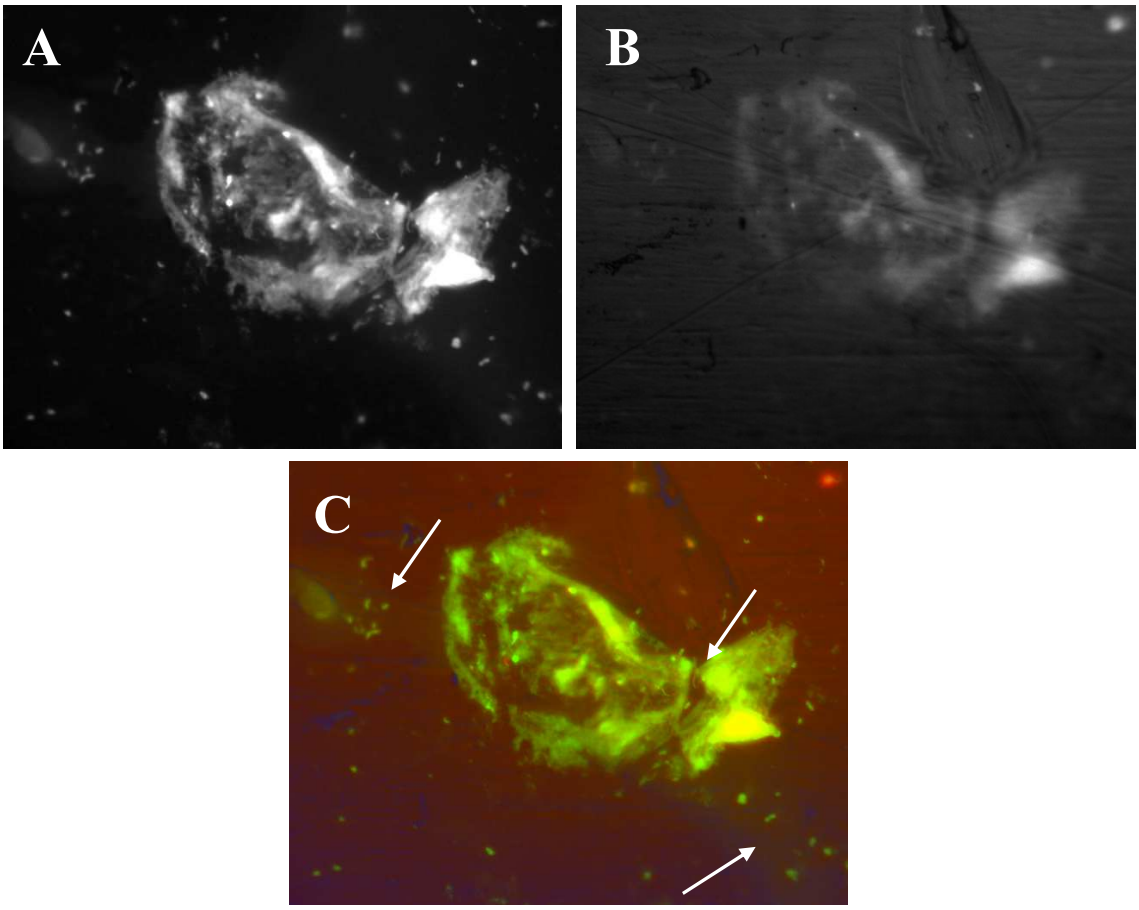


Fig. 2.8. EF-1 image of a biofilm at a coupon removed from the second vessel 5 days after the inoculation with *H. pylori*. Visualization under the: GFP filter (a); Rodhamine filter (b); and false-color superimposition of the previous two pictures (c). Some of the positive *H. pylori* are indicated with arrows.

Spiral forms of the bacteria, ranging from 2 to 4 μm long were still identified five days after the spiking of the chemostat. Coccal and U-shaped cell morphologies were also detected throughout the experiment. All types exhibited a strong signal with the 16S rRNA PNA probe in the green channels of both microscopes. These data indicate that there was a high content of RNA and ribosomes in *H. pylori*, and that all morphological

types of cells were still viable after 5 days in the biofilm. It has been suggested that, contrary to most eubacterial mesophiles, *H. pylori* exhibits a relaxed phenotype with respect to accumulation of 16S rRNA under adverse conditions (218). Therefore, it is not clear if this strong signal implies a metabolically active form of the cell or simply the accumulation of ribosomal RNA in inactive cells.

The fact that spiral forms were still detected after 5 days may be of great importance to public health, because spiral shaped bacteria are usually associated with a more viable form of the bacteria (Section 1.2.3). This might suggest that the pathogen can survive, at least transiently, in mains water supplies and pose a risk of waterborne infection to the human population. This situation would be exacerbated if the biofilm provided some protection against routine chlorination practice, providing a safe haven for the pathogen.

It is notable that *H. pylori* could still be easily identifiable by both PNA and characteristic morphology in the basal layer of the biofilm, especially close to the stacks. This suggests that like *Legionella pneumophila* and *Campylobacter jejuni* it is a motile microaerophile which migrates to low redox zones in heterogeneous biofilms (111).

This study represented the first reported use of PNA oligonucleotides for *in situ* detection of microorganisms in biofilms. The PNA technology seems to be easily adapted to the study of pathogens in biofilms, and offers a more efficient and faster alternative conventional DNA approaches. In the future, further exploration of the PNA special features, might allow the simultaneous detection of different microorganisms in the same sample.

2.3.5 Detection of *H. pylori* in real drinking water distribution systems

Hybridization of the pipeline coupons was done with the same protocol as for the glass slide smear tests. Putative *H. pylori* was found in one of the samples from Riga (Fig. 2.9), but no *H. pylori* was detected in the Yorkshire or Minho pipe samples.

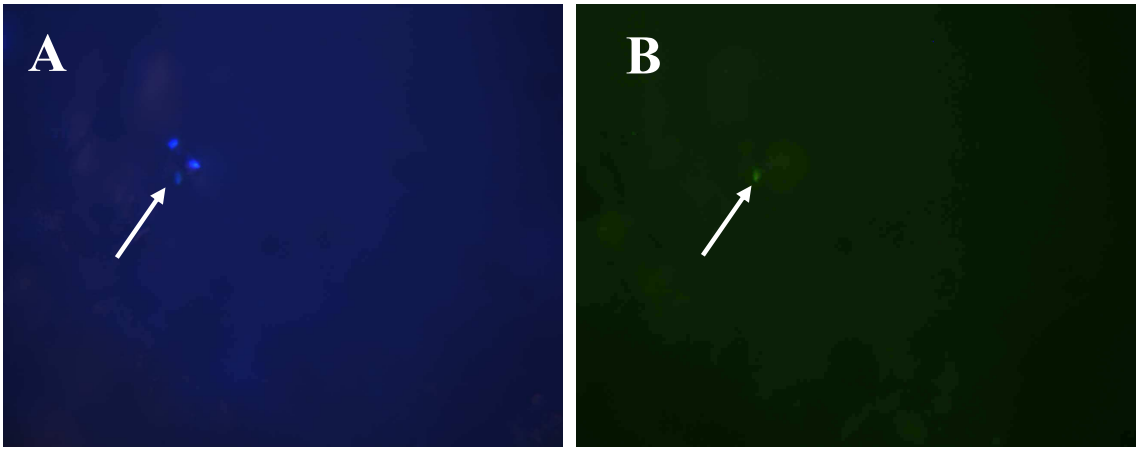


Fig. 2.9. EF-2 image of a section of the cast iron pipe from Riga observed in the DAPI (a) and LIVE/DEAD (b) channels. The putative *H. pylori* is indicated with an arrow (strong green and medium blue fluorescence).

Although the results might reflect the low prevalence/nonexistence of the bacteria in real pipes, they can also be caused by some of the problems encountered when adapting the PNA FISH method to pipes from real distribution systems. These problems, together with some suggested ways to circumvent them, are described as follow:

i) Pipe curvature: Pipes with small diameters have large curvatures, which makes the task of focusing in the right plane difficult. This problem can be minimized by choosing pipes with very large diameters (such as the pipe from Riga). However, if it is intended to monitor every type of situation that occurs in distribution networks, including low diameter pipes, it might be necessary to install an *in-situ* biofilm formation device. This device should have at least one flat wall, such as the flow cell reactor (191).

ii) Pipe texture/deposits: Adding to the focus plane problem of the last point, the material itself might be so irregular, that focusing is nearly impossible. This is the case of cement. Deposits formed on the surface of other materials, such as cast iron, may also add to problems of focusing (Fig. 2.10). These problems can be partially overcome by using the EDIC/EF microscope. Deposits can also be scraped, filtered and observed on the filter membrane surface, although in this case the 3-D information is lost.

Fig. 2.10. Deposits formed in a 20 year old pipe of cast iron from Minho. Analysis of such heavily fouled pipes is only possible after scraping and filtering of the deposits.

iii) Autofluorescence: Originally it was thought that, because this condition has been rarely reported before, autofluorescence would not play a significant role in most detection works carried out *in situ*. However, and as with lab-grown biofilms, it was observed that autofluorescence was present in all samples, although for cast iron and HDPE it only appeared at certain sections of the pipes. Other substrata, such as cement, presented such a strong fluorescence associated with the material itself that PNA FISH analysis was impossible to perform. As several other European groups, with whom there is a cooperation under an European Commission-funded project named SAFER, have now reported the same problem (unpublished results), autofluorescence appears to be universal when analyzing drinking water biofilms. It can occur due to the composition of the pipe material, autofluorescent microorganisms and chemical or biological deposits on the surface. Methods to tackle this problem have already been discussed in Section 2.3.2.

iv) Looking for a needle in a haystack: During the lab experiments, a large quantity of microorganisms is spiked into the particular model system. This makes the certainty of the detection much higher, because a large number of microorganisms appear on the coupon after spiking, which can be compared with a negative control. Finding only one or a group of a certain microorganism, which might not even be there, is a much more challenging task. This is what really makes the optimization of the former parameters essential.

Only after solving these problems will the assessment of the prevalence of *H. pylori* in European drinking water systems be deemed trustworthy. The existence of autofluorescing stacks and microorganisms means that practice in microscopy visualization is essential for the correct detection of the PNA-labelled pathogen in these samples. Measures to resolve some of the problems have been already taken, such as the installation of two flow cells in a water supply in Minho region, or the design of a new probe labeled with an Alexa Fluor dye.

Besides this work, only Moreno *et al.* was capable of detecting *H. pylori* in non-inoculated water samples using FISH technology (163). Because sampling was performed in river and wastewater samples, one of the possible reasons this screening might have been successful was possibly the lower amount of autofluorescence-emitting substances when compared to biofilm samples. This study, together with other studies using the PCR technique (123, 186, 206, 259), appear to indicate that *H. pylori* can indeed be commonly found in real water systems, which implies that the low prevalence of the pathogen found in this study is mainly determined by the technical problems that were encountered.

2.4 Conclusions

With the work described in this Chapter it was intended to assess the ability of *H. pylori* to attach and integrate in a heterotrophic biofilm formed by endogenous species of drinking water systems. To achieve that goal, a PNA FISH probe was designed and tested for its suitability and specificity to detect the pathogen in those structures. Using that new technology, it was shown here that *H. pylori* can successfully incorporate into lab-grown biofilms and that the different morphological forms can subsist and be detected at least for five days after inoculation, bringing new concerns to water supply public health policy. These concerns are amplified by the subsistence of *H. pylori* close to the basal layer of the biofilm, which suggests that the bacterium is able to migrate to low redox zones created by these structures. However, until detection in real drinking water systems is consistently accomplished by this method, doubts about the prevalence and survival of the microorganism in the environment will remain to be raised. For that, it is essential to solve the problems described earlier, especially the problem caused by autofluorescence.

3 Factors affecting the attachment of *H. pylori* to abiotic substrata

We have shown in Chapter 2 that *H. pylori* could attach to drinking water heterotrophic biofilms, and that it could be located both directly attached to the surface and in the basal layer of frond or stacks. In this Chapter, factors that are known to affect the formation of both heterotrophic and monospecies biofilms were evaluated for their influence in *H. pylori* attachment. Additionally, the thermodynamic theory of adhesion was assessed for its suitability to explain the adhesion of *H. pylori* to different substrata. Physiological assessment of attached bacteria using fluorochromes and standard plating culture techniques was also performed.

3.1 Introduction

In the last years, the concept that a limited number of microbial species were 'good biofilm formers' has been supplanted by the sense that biofilm formation is a universal feature of microbes (121). *H. pylori* was shown to also obey that rule when Stark *et al.* demonstrated that the pathogen was capable of forming a monospecies biofilm and producing polysaccharides as a component of the biofilm matrix (228). Recently, Cole *et al.* described the formation of a *H. pylori* biofilm with three dimensional structure at the air-liquid interface in stationary or shaking batch cultures (41). However, these studies have been conducted under high nutrient conditions, which did not model the environment that *H. pylori* would encounter in a water distribution network. Because much of the research on *H. pylori* transmission in water is focused in detecting the bacterium in drinking water and associated biofilms, it is critical that a detailed description and explanation of the factors affecting the attachment of the bacterium to plumbing materials under low nutrient conditions is executed. This information will allow a more rationale selection of locations to perform molecular or plate culture analysis for the detection of *H. pylori* in water supplies and provide hints for possible extended survival of the bacteria under those conditions.

3.1.1 Factors affecting the formation of biofilms

Several parameters are known to govern biofilm accumulation and architecture in drinking water distribution systems and other water supplies (155). These include temperature, pH, shear stress, chlorine and other biocidal agents concentration, nutrient concentration, microbiological profile and concentration and plumbing materials. The application of chlorine, which is probably the most common way to control water quality, has been intensively used for nearly one hundred years in the more developed countries and has virtually wiped out large scale epidemic diseases. On the other hand, high nutrient concentrations have been shown to promote bacterial growth in biofilms and therefore deteriorate water quality. As temperature, shear stress, microbiological concentration and different substrata are the parameters evaluated in this work, a more detailed discussion of their effect will follow.

Temperature

Microorganisms tend to have temperature ranges at which their growth is optimized. Temperatures higher than this range will cause protein denaturation and consequent malfunctioning of the cell, while lower temperatures provoke reduced protein activities. Because optimal growth temperature is largely dependent on the type of microorganism, it will affect the microbiological profile of the biofilm consortia as well as physiology of individual cells. Despite this expectable effect, Momba *et al.* and Ollos *et al.* have independently reached the conclusion that temperature was a much less important factor than the chlorine concentration or the biodegradable matter concentration in biofilm formation (162, 177). This apparent lack of importance is easily explained by the fact that these studies were conducted by analyzing heterotrophic consortia, where several groups of microorganisms preferring different temperatures of growth prevail according to the microenvironmental conditions, overshadowing negative effects that temperature has in other groups of microorganisms. For individual microorganisms or small groups of closely related bacteria, temperature does appear to play an important factor in the attachment and survival (28, 204). As water temperatures in drinking water distribution systems of Northern Europe are usually below 15 °C, while in the Mediterranean countries can be over 20 °C during the summer months, different consortia of microorganisms are expected in each of these areas.

Shear stress

The most noticeable effect of shear stress in biofilms is in their structure, as biofilms formed under turbulent conditions are more compact and with a less porous structure to withstand the physical stress caused by the movement of the water (58, 96, 258). On the one hand this might implicate a more difficult mass transfer inside the structure, whilst on the other hand the turbulence caused by the higher fluid velocity decreases biofilm height and increases mass transfer to provide increased nutrient supply for a denser biofilm. As reported in Pereira *et al.* (191), the specific respiratory activity of bacteria is higher for turbulent flow regimes, possibly due to a more efficient mass transfer of nutrients and

oxygen into the biofilm structure, which might explain why the percentage of culturable cells is also higher for this type of flow regime. Higher numbers of total bacteria are usually obtained as the shear stress increases (37, 190), suggesting that cells tend to live a sessile existence as a sheltering mechanism from the harmful physical stress caused by water. However, this factor has been shown to be less important than the nutrient concentration or disinfectant residual in the formation of heterotrophic biofilms (37, 177).

Microbiological concentration in the planktonic state

Kerr *et al.* observed that the numbers of planktonic bacteria in the effluent water leaving different biofilm formation systems directly correlated with the numbers in the biofilm phase on each of the systems (116). Other authors have also indicated that a direct correlation between planktonic bacteria in the system and bacteria in biofilms could be achieved (18, 246). It is believed that what causes this correlation to occur is the sloughing off from the biofilms into the water. Deviations to these correlations appear when occasional contamination of the water flowing in the pipes by external sources occurs.

Support material

Support material has been one of the most thoroughly studied factors in biofilm formation. It is well known that every material used in drinking water distribution systems supports the attachment of microorganisms, but the extent of that attachment depends on the leaching of nutrients required for bacterial growth that is a characteristic of each material (161, 262). Momba *et al.* and Rogers *et al.* reported that cement-based and copper surfaces materials, respectively, significantly supported less fixed bacteria than plastic-based materials (162, 204). Kerr *et al.* concluded that cast iron supported higher diversity and total counts of heterotrophic bacteria than polyethylene and polyvinyl chloride (PVC) (116), but other authors could not conclude as to whether stainless steel (SS) or PVC would support more biofilm growth (188, 268). Pipe material influence also ranked last when assessing the relative importance of nutrients, shear stress and pipe material (PVC vs. SS) in total and culturable counts of heterotrophic bacteria in a biofilm (37). In general, cement-based materials and copper appear to support less biofilm growth than

other metallic or plastic-based materials, but the effect of pipe material in heterotrophic biofilms might be comparable to temperature in the sense that different groups of microorganisms might proliferate depending on the type of substrata used.

3.1.2 Interaction forces of microbial adhesion to inert substrata

Although microbial adhesion to inert substratum surfaces is frequently described in terms of specific interactions between specific molecular groups (such as adhesins), it is important to realize that all interaction forces originate from the same fundamental forces, including the Lifshitz-van der Waals and electrostatic forces, and acid-base interactions (20). Mathematical models have been developed to estimate these forces or interactions, based on measurements of the surface tension (or energy) and its different components (polar, apolar, electron donating and electron accepting) of the bacterial cell surface and/or the solid substrate. Most notably, two physico-chemical theories, the thermodynamic and the DLVO (named after Derjaguin, Landau, Vervey and Overbeek who developed it during the 1940s), initially considered distinctly different, are available to describe these interactions (20). Both approaches have proven merits for microbial adhesion when certain collections of strains and species are considered, but have failed so far to yield a generalized description of all aspects of microbial adhesion valid for each and every strain (250).

In practical terms, surface tension can be determined using different independent methods, such as direct force measurement, contact angles, capillary penetration into columns of powders or sedimentation of particles, but the contact angle is believed to be the simplest and hence the most widely used (220). Contact angles describe the shape of a small drop of liquid in contact with a solid surface. The drop will spread out until the liquid cohesion (the attraction molecules of one material feel toward other molecules of the same material) is balanced by its adhesion to the solid.

Using contact angle measurements, surface tension is able to be calculated by several approaches, including the Equation of state, Geometric mean or Lifshitz-van der Waals acid-base (LWAB) approaches (Fig. 3.1). It has been however concluded that the LWAB was more consistent when compared to other methods (43, 67, 220). This approach uses

three different liquids (2 polar and 1 apolar) to calculate the free energy of adhesion, and was firstly described by van Oss in 1995 (249). This is still a very active research field where new and improved methodologies are being developed constantly. For instance, just one year ago Combe *et al.* suggested that liquid triplets could still yield misleading estimates of the components of surface tension and suggested a testing protocol in which up to six test liquids should be used (43). Because the thermodynamic theory using the Lifshitz-van der Waals acid-base (LWAB) approach will be the one used in this work, it will be subsequently characterized in detail.

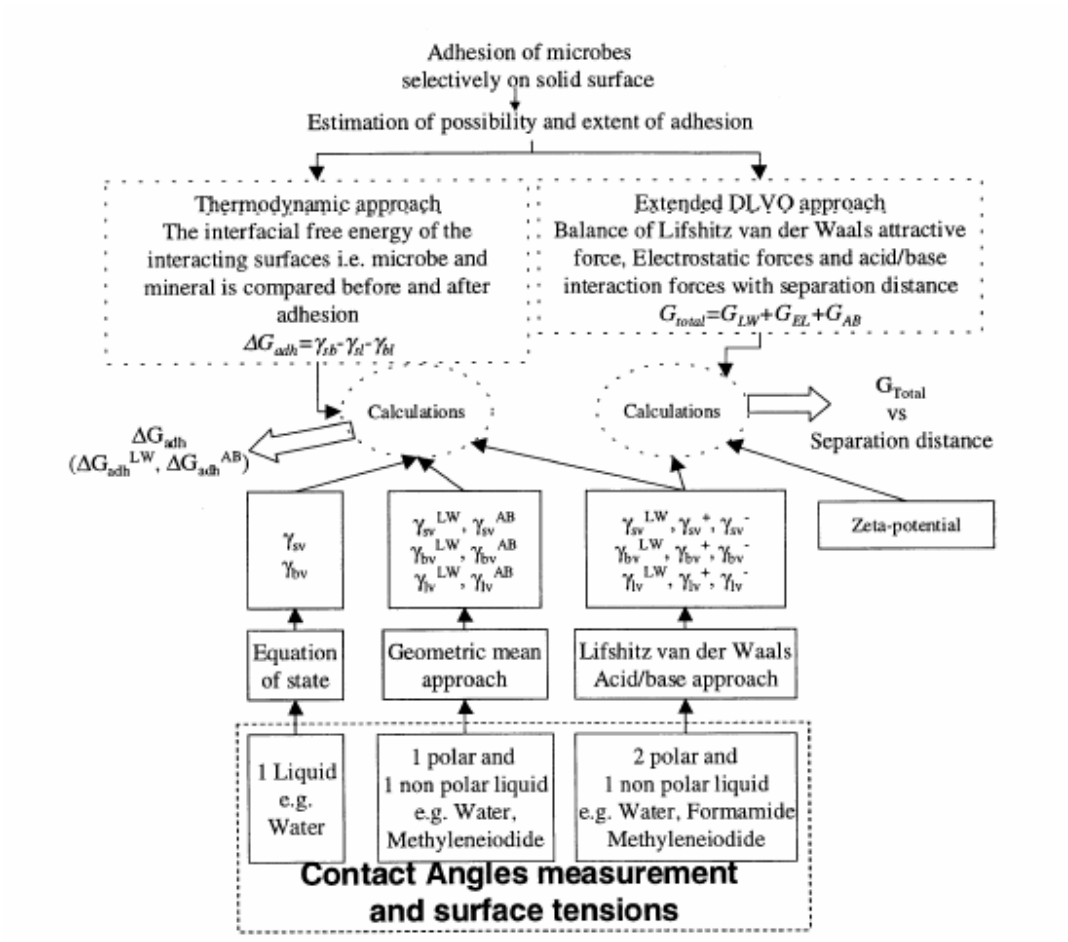


Fig. 3.1. Theoretical estimation of the possibility and extent of microbial adhesion on solid surfaces. Approaches to convert contact angle data into solid surface energy. Adapted from Sharma and Rao (220).

Lifshitz-van der Waals acid-base (LWAB) approach

In the thermodynamic approach towards microbial adhesive interactions, the interfacial free energies between the interacting surfaces are compared. Accordingly, this comparison is expressed in the so-called free energy of adhesion

$$\Delta G_{adh} = \gamma_{sm} - \gamma_{sl} - \gamma_{ml} \quad \text{(Equation 1)}$$

where ΔG_{adh} is the free energy of adhesion, and γ_{sm}, γ_{sl}, and γ_{ml} are the solid (s)-microorganism (m), solid-liquid (l), and microorganism-liquid interfacial free energies, respectively. Like all systems in nature, the system of interacting microorganism and solid surfaces will also strive to obtain a state of minimal free energy. Therefore, microbial adhesion is favorable to occur from a free energy point of view, when ΔG_{adh} is negative, while adhesion is energetically unfavorable when ΔG_{adh} >0 (20).

Because the LWAB approach states a clear subdivision between apolar (LW) and polar (AB) interactions, it is possible to arrive at a quantitative definition of the free energy of adhesion

$$\Delta G_{adh} = \Delta G_{slm}^{LW} + \Delta G_{slm}^{AB} \quad \text{(Equation 2)}$$

where ΔG_{slm}^{LW}, is the apolar energy of adhesion of the solid-liquid-microorganism system, and ΔG_{slm}^{AB} the polar counterpart. Similarly to the free energy of adhesion, the polar and apolar energy of adhesion in this system are expressed by:

$$\Delta G_{slm}^{LW} = \gamma_{sm}^{LW} - \gamma_{sl}^{LW} - \gamma_{ml}^{LW} \quad \text{(Equation 3)}$$

$$\Delta G_{slm}^{AB} = \gamma_{sm}^{AB} - \gamma_{sl}^{AB} - \gamma_{ml}^{AB} \quad \text{(Equation 4)}$$

For a binary system of interaction (for instance solid-microorganism), the interfacial free energies can be calculated by

$$\gamma_{sm}^{LW} = \gamma_{s}^{LW} + \gamma_{m}^{LW} - 2 \times \left(\sqrt{\gamma_{s}^{LW} \times \gamma_{m}^{LW}} \right) \quad \text{(Equation 5)}$$

$$\gamma_{sm}^{AB} = 2 \times \left(\sqrt{\gamma_{s}^{+} \times \gamma_{s}^{-}} + \sqrt{\gamma_{m}^{+} \times \gamma_{m}^{-}} - \sqrt{\gamma_{s}^{+} \times \gamma_{m}^{-}} - \sqrt{\gamma_{s}^{-} \times \gamma_{m}^{+}} \right) \quad \text{(Equation 6)}$$

where γ_s^{LW} and γ_m^{LW} represent the Lifshitz van der Waals interactions for both components and γ_s^+, γ_s^-, γ_m^+, γ_m^- represent the electron-acceptor and electron-donor parameters of acid–base component of the surface tension for the solid and the microorganism. For a few liquids these values are already tabulated (Table 3.1), and that information can be used to determine solid surfaces or microbial lawns parameters by contact angles. In mathematical terms, this can be achieved by combining the equation of Young together with an equation of state that relates the different interfacial free energies. In the LWAB theory, the final equation appears in the form of

$$\gamma_l(1+\cos\theta) = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right) \quad \text{(Equation 7)}$$

where $\cos\theta$ is the angle between a liquid with known properties and the solid surface of interest. Equation 4 still contains three unknowns, γ_s^{LW}, γ_s^+ and γ_s^-, and therefore requires contact angle measurements with at least three different liquids, 2 polar and 1 apolar. A more detailed description of the theories subjacent to microbial adhesion can be found in (37) and in (220).

Table 3.1. Surface tension components and parameters (mJ/m^2) of liquids often employed in contact angle measurements. Adapted from Bos *et al.* (20).

Liquid	γ_l	γ_l^{LW}	γ_l^+	γ_l^-
Water	72.8	21.8	25.5	25.5
Glycerol	64	34	3.92	57.4
Ethylene Glycol	29	48.0	1.92	47.0
Formamide	58	39	2.28	39.6
α-Bromonaphthalene	44.4	44.4	-	-
Diiodomethane	50.8	50.8	-	-

A particular case of this theory can be applied to solid surfaces immersed in water to calculate the degree of hydrophobicity (249). This concept has been, according to van der Mei (245), the most studied property of the cell surface with regard to their adhesion to

surfaces. The hydrophobicity, defined as the free energy of interaction between molecules of a solid surface (s) immersed in water (w), is calculated in analogy to Equation 2 as:

$$\Delta G_{sws} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB} \quad \text{(Equation 8)}$$

Combining Equation 3 and 5, as well as Equation 4 and 6, the polar and apolar energy of adhesion in this system are expressed by:

$$\Delta G_{sws}^{LW} = -2 \times \left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}} \right)^2 \quad \text{(Equation 9)}$$

$$\Delta G_{sws}^{AB} = -4 \times \left(\sqrt{\gamma_s^+ \times \gamma_s^-} + \sqrt{\gamma_w^- \times \gamma_w^+} - \sqrt{\gamma_s^+ \times \gamma_w^-} - \sqrt{\gamma_s^- \times \gamma_w^+} \right) \quad \text{(Equation 10)}$$

Hydrophobicity informs about the affinity of a solid surface or a microorganism to water and is therefore particularly useful to understand the behaviour of a bacterium in drinking water distribution systems.

3.2 Material and methods

3.2.1 Culture maintenance

H. pylori NCTC 11637 obtained from PHLS (Colindale, UK) was mantained on Columbia Agar (Oxoid) supplemented with 5% (v/v) defribinated horse blood (Biomérieux, Marcy l'Etoile, France) in the presence and absence of 1% (v/v) fetal calf serum (Merck, Germany), hence denominated CBA. Plates were incubated at 37 ^{o}C in a Genbox jar 2.5 L (Biomérieux) under microaerophilic conditions created using a Genbox microaer sachet (Biomérieux) and streaked onto fresh plates every 2 or 3 days.

3.2.2 Test surfaces preparation

Coupons measuring 2×2 cm were prepared at the Centro de Engenharia Biológica from stainless steel 304 (SS304; F. Ramada, Ovar, Portugal) and 316 (SS316; F. Ramada), copper (Companhia Portuguesa do Cobre, Porto, Portugal), polyvinyl chloride (PVC; Neves e Neves, Trofa, Portugal), polypropylene (PP; Vimágua, Guimarães, Portugal) and glass (slides 75×25mm, Moreira Costa e Santos, Porto, Portugal). Copper coupons were polished with an alumina suspension (Struers, Copenhagen, Denmark). All materials were

immersed in a solution of 5% (v/v) commercial detergent (Top Neils, Tengelmann Portugal, Sintra, Portugal) and pre-warmed distilled water for 30 min while gently mixed. To remove residual detergent, coupons were rinsed 5 times in ultrapure water, air dried and wrapped in foil. They were subsequently immersed in 90% (v/v) ethanol for 30 min, except PVC and PP that were only immersed for 10s. After being rinsed with ultra-pure water, air dried and wrapped in foil, SS304, SS316, copper and glass coupons were autoclaved for 15 min at 121 ^{o}C, whereas PP and PVC were heated for 20 min at 80 ^{o}C. Each coupon was finally placed in a well of 6-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium).

3.2.3 Exposure of the test surfaces to an *H. pylori* suspension

This study analyzed the influence of shear stress, temperature, inoculum concentration and different substrata in the attachment of *H. pylori*. For all conditions, cells from 2 days old cultures were harvested from Columbia Agar plates, suspended in 10 ml of autoclaved distilled water and vortexed for 30s. This suspension served as a primary inoculum.

Shear stress and temperature influence

The primary inoculum was transferred to a sterile flask containing 1000ml of autoclaved distilled water, to achieve a final concentration of approx. 10^6 CFU ml^{-1}. The flask was maintained at room temperature (approx. 23±2ºC) and continuously stirred (120 rpm) using a magnetic bar, to ensure the necessary homogenization. After 10 minutes, 5ml of the flask were dispensed into each of the wells of 6-well tissue culture plates containing SS304 coupons. The tissue culture plates were then either placed in orbital incubators (Certomat® S, B. Braun Biotech International, Germany) set to 23ºC and under various shear forces (0, 60 and 120 rpm), or at various temperatures (4, 23 and 37ºC) and no rotation.

Inoculum concentration influence

The primary inoculum was transferred to three different sterile flasks, to achieve a final concentration of either 6.5×10^5, 2.5×10^6 and 6.9×10^6 CFU ml^{-1}. The flasks were

maintained at room temperature (approx. 23±2ºC) and continuously stirred (120rpm) using a magnetic bar. After 10 minutes, 5ml of each of the flasks were dispensed into each of the wells of 6-well tissue culture plates containing SS304 coupons. The tissue culture plates were then placed at 23ºC and no rotation.

Substrata influence

The primary inoculum was transferred to a sterile flask containing 1000ml of autoclaved distilled water, to achieve a final concentration of approx. 10^6 CFU ml^{-1}. The flask was maintained at room temperature (approx. 23±2ºC) and continuously stirred (120rpm) using a magnetic bar. After 10 minutes, 5ml of the flask were dispensed into each of the wells of 6-well tissue culture plates containing SS304, SS316, copper, PP, PVC or glass coupons. The tissue culture plates were then placed at 23ºC and no rotation.

For all experiments and at several times of exposure (2, 6, 12, 24, 48, 96 and 192h), one or more coupons of each condition were removed from the well, rinsed three times in autoclaved distilled water, and left to air dry. Coupons were then used to perform culturable and total cell counts, assessing membrane integrity and for visualization under a scanning electron microscope (SEM).

3.2.4 Culturable cell counts of planktonic and sessile *H. pylori*

H. pylori concentration on the flasks was determined by surface plating 100 µl of the appropriate dilutions (1:10 dilutions in distilled water) onto three to five plates of *H. pylori* special peptone agar (HPSPA) (233). Plates were incubated at 37 ºC for 6 days under the same microaerophilic conditions used for culture maintenance.

For the study of substrata influence, CFU of planktonic *H. pylori* on the wells containing SS304 coupons was analyzed temporally by plating an appropriate volume on to HPSPA plates. In the same study, three methods were applied to detach *H. pylori* from all types of substrata: vortexing with glass beads, scraping and sonication. In all cases coupons were immersed in 10 mL of sterile distilled water. Vortexing was carried out with 1 mm diameter glass beads (Sigma) for 30s in a Heidolph Reax 2000 (Heidolph Instruments

GmbH & Co. KG, Schwabach, Germany). Scraping was performed systematically for 10 times in each direction, and sonication was undertaken for 3×1min bursts with 10s interval and 10% amplitude (GEX 400 Ultrasonic Processor; Sigma). Then, 100 μL of the distilled water containing detached cells were dispensed on HPSPA plates and incubated microaerophilically at 37 ºC for 6 days to determine the numbers of culturable *H. pylori*.

3.2.5 Total cell counts of planktonic and sessile *H. pylori*

Total cell counts of planktonic bacteria were obtained by filtering 100-200 μL of the suspension in the well through a 25 mm black Nuclepore® polycarbonate membrane with a pore size of 0.2 μm (Whatman, Kent, U.K.). After filtration, cells on the membrane were stained with 100 μg/mL 4,6-diamino-2-phenylindole (DAPI) (Sigma) for 5 min. The membranes were then rinsed in distilled water, left to air-dry and a drop of mounting oil added to their surface. Finally, membranes were covered with a coverslip and stored in the dark for up to 4 days. No significant decay of fluorescence was noticed during this time span. Cells were visualized using the EF-3 microscope (see Table 2.3) with the filter set sensitive to DAPI fluorescence. A total of 20 fields were counted using an ocular grid and the average was used to calculate total cells per mL of sample. Depending on the number of quadricules of the ocular grid considered, the area of each field counted ranged from 0.04 to 0.25 mm^2. For smoothing and analysis purposes, results were transformed to $\log_{10}$ total cells per mL of sample.

Total cell counts of adhered bacteria were obtained by a similar method, but 80 μL of the DAPI solution was applied directly to the substrata which were then covered with a coverslip. For each coupon, 50 fields were counted and the results expressed as $\log_{10}$ total cells per cm^2. At selected times, five fields were used to determine the proportion of spiral/coccoid bacteria either in the planktonic phase or when attached to the different materials.

3.2.6 Live/Dead staining procedure

To assess the membrane integrity of the sessile bacteria, the LIVE/DEAD BacLight kit (Molecular Probes, Oregon, U.S.A.) was used (21). The two reagents (i.e. Syto9 and

propidium iodide) were prepared according to the manufacturer instructions, and mixed in equal proportions. The mixture was then applied to coupons of SS304 and copper (in the substrata influence experiment) with different times of exposure (20 µl per coupon), covered with a coverslip and incubated for 15 min in the dark. Coverslips were then removed, the coupon allowed to air-dry in the dark, and a drop of mounting oil added to the surface. A new coverslip was placed and the cells were immediately visualized under the EF-3 microscope on the filter set that simultaneously detected the two components of the mixture.

3.2.7 Scanning electron microscopy

For the dehydration procedure, coupons with 192h and 2 months of exposure to *H. pylori* were immersed for 15 minutes in solutions with increasing concentrations of ethanol up to 100% (v/v), and placed in a sealed desiccator. The test surfaces were mounted on aluminium stubs with carbon tape, sputter coated with gold and observed with a Leica Cambridge S-360 SEM (Leo, Cambridge, UK). For the substrata influence experiment coupons of copper and SS304 with two months exposure-time were also analyzed. The scanning electron microscope was equipped with an X-ray analyzer, which allowed identification and quantification of certain components on the surface of the materials.

3.2.8 Contact angle adhesion assay

The preparation and exposure of test surfaces to an *H. pylori* suspension were performed as described in the previous sections. The tissue culture plates containing the coupons were placed at 23ºC and no rotation, and three coupons of each material were taken after 48h of exposure for total cell counts of *H. pylori*, as described in Section 3.2.5.

3.2.9 Contact angle measurement on test surfaces and microbial lawns

Contact angles were performed by the sessile drop technique on the test surfaces treated as described in the test surface preparation section, using a contact angle measurement apparatus (model OCA 15 PLUS, DataPhysics Instruments GmbH, Filderstadt, Germany). Images of the drop were acquired with an incorporated video camera and the contact angle

was measured using SCA-software. The measurements were performed at room temperature, using three different liquids: water, formamide and 1-bromonaphthalene (all from Sigma). Replacing the values for the three chosen liquids from Table 3.1 into equation 7, the following equations are obtained

$$5.049\times\sqrt{\gamma_s^+}+5.049\times\sqrt{\gamma_s^-}=36.4\times(1+\cos\theta_W)-15.550\times(1+\cos\theta_B) \qquad \text{(Equation 11)}$$

$$6.293\times\sqrt{\gamma_s^+}+1.510\times\sqrt{\gamma_s^-}=29.0\times(1+\cos\theta_F)-20.806\times(1+\cos\theta_B) \qquad \text{(Equation 12)}$$

$$\gamma_s^{LW}=11.1\times(1+\cos\theta_B)^2 \qquad \text{(Equation 13)}$$

where θ_W, θ_B and θ_F are the contact angles formed between the surface and a drop of water, 1-bromonaphthalene and formamide.

Each type of substrata was evaluated in triplicate and at least 10 contact angles per surface and per liquid were measured. These results were compared with the results obtained with test surfaces not subjected to treatment.

In the preparation of *H. pylori* microbial lawns, as described in Busscher *et al.* (31), 150 mL from the same *H. pylori* suspension that served to inoculate the test surfaces were filtered through 47 mm diam., 0.2 μm pore size membranes (Pall Gellman, New York, U.S.A.). Membranes were subsequently sliced in four parts and left to dry in a Petri dish containing glycerol agar. This drying step on glycerol agar serves two purposes - agar acts as a moisture buffer for approximately 3.5 h and it does not allow the filter to dry, secondly the moisture content in all the filters with bacterial lawns are brought to the same level before they are dried under controlled conditions, as the moisture content left after the filtration process may not be the same (220). Values obtained were subsequently compared with the experimental values of adhered cells obtained by fluorescence microscopy.

3.2.10 Confirmative procedures and analysis of data

Besides checking for typical colony morphology (i.e., round, translucent to yellowish, convex, 0.2-2 mm diameter) (4), hybridization with the peptide nucleic acid probe described in the previous Chapter was performed to confirm the identity of *H. pylori*.

For smoothing purposes, total cell counts were transformed to the $\log_{10}$ scale. Steady state was considered to have occurred after 48h, and averages were calculated based on the subsequent three values for each case. Averages were then compared using an appropriate statistical package (SPSS Inc., Chicago, U.S.A.) to perform a one-way ANOVA followed by a Bonferroni or LSD *post hoc* test. For the contact angles assay, the same analysis was performed on the three coupons of each material tested after 48h. Results were considered statistically relevant if $P<0.05$.

3.3 Results and discussion

3.3.1 Evaluation of the DAPI method for the quantification of *H. pylori*

The DAPI method allowed an efficient discrimination and quantification of the adhered *H. pylori* with nearly all different substrata (Fig. 3.2). One of the exceptions was copper, where fluorescence was at times very faint. When studying biofilm formation on different substrata, quantification was possible to obtain on copper mainly because the sample was analyzed immediately after the staining procedure. However, and because no such care was taken in the contact angles adhesion assay, the results obtained there were considered unreliable. Originally, analysis on cement coupons was also intended but, as referred in the previous Chapter, the high level of autofluorescence associated to the material prevented quantification with this method.

DAPI also allowed the identification of all different morphological forms (spiral, U-shaped and coccoid) of the bacteria, with coccoid forms usually fluorescing more intensely than spiral or U-shaped forms. For the studies concerning the impact of shear stress, temperature and inoculum concentration, there were no observable changes in the fluorescence intensity for any of the conditions tested. Lower fluorescence intensity using the DAPI method has been found for chlorine and nutrient-stressed cells (37, 212). In this case however, total cell counts of attached bacteria as well as morphology prevalences could easily be determined.

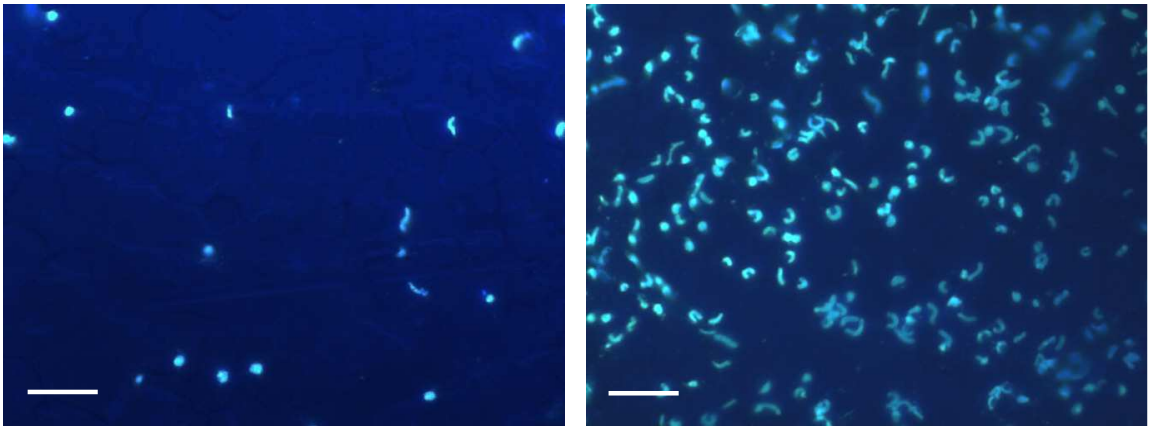


Fig. 3.2. Fluorescence microscopy image showing the adhesion of *H. pylori* to SS304 after 6 and 192h at 23°C and no shear stress. Bar represents 10 μm.

3.3.2 Influence of temperature, shear stress and inoculum concentration in the adhesion of *H. pylori*

Total cell counts of attached *H. pylori* obtained for the different experiments and conditions are shown in Fig. 1. After only two hours, more than 5 $\log_{10}$ total cells cm^{-2} were found to be attached, regardless of the conditions tested. Temperature appeared to have no effect on the attachment of the pathogen (Fig. 3.3.a), with most experimental points with time overlapping for the three temperatures and increasing up to 6.5 $\log_{10}$ total cells cm^{-2} after 192 hours. Decreasing shear forces, however, clearly promote the attachment, with an observable difference noticeable from the beginning of the experiment (Fig. 3.3.b). Increasing inoculation densities appear to have a proportional effect on the attachment at the initial stages of the experiment, but that effect appeared to disappear for the later stages of the experiment, as the attachment reached also approx. 6.5 $\log_{10}$ total cells cm^{-2} for all conditions at the end of the experiment (Fig. 3.3.c).

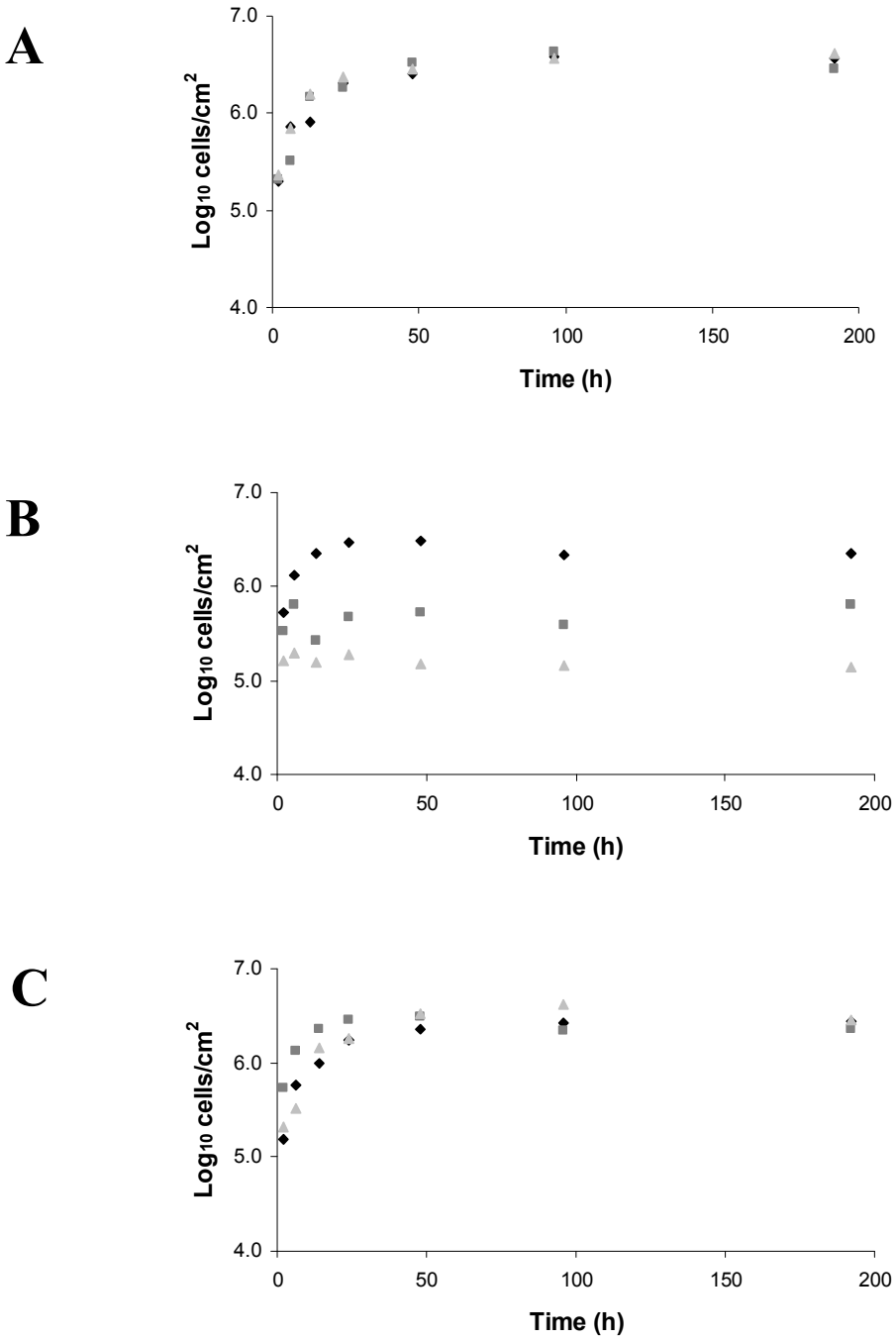


Fig. 3.3. Influence of different conditions on the adhesion of *H. pylori* (NCTC 11637) to SS304. a) Temperature (♦ 4 °C; ▲23 °C; ■ 37 °C). b) Shear stress (♦ 0 rpm; ■ 60 rpm; ▲ 120 rpm) and c) Inoculation concentration in $\log_{10}$ CFU ml^{-1} (▲ 5.8; ♦ 6.4; ■ 6.8).

To obtain a more clear view and with statistical significance, the average of the last three values of each condition was determined and the results plotted (Fig. 3.4). One-way ANOVA followed by Bonferroni analysis confirmed that, for different shear stresses, averages are statistically significant between any of the combinations possible ($P<0.001$ in all cases). Consequently, another study on the adhesion of *H. pylori* to PP at different shear stresses was also performed in our lab. Similarly to SS304, attachment of *H. pylori* at 120 rpm and 60 rpm represented only 77% and 89%, respectively, of the attachment obtained at 0 rpm. Even though only two coupons for each condition were tested, results are still statistically significant at the $P<0.05$ level. No conclusions could be drawn about the effect of temperature or inoculation concentration ($P>0.05$).

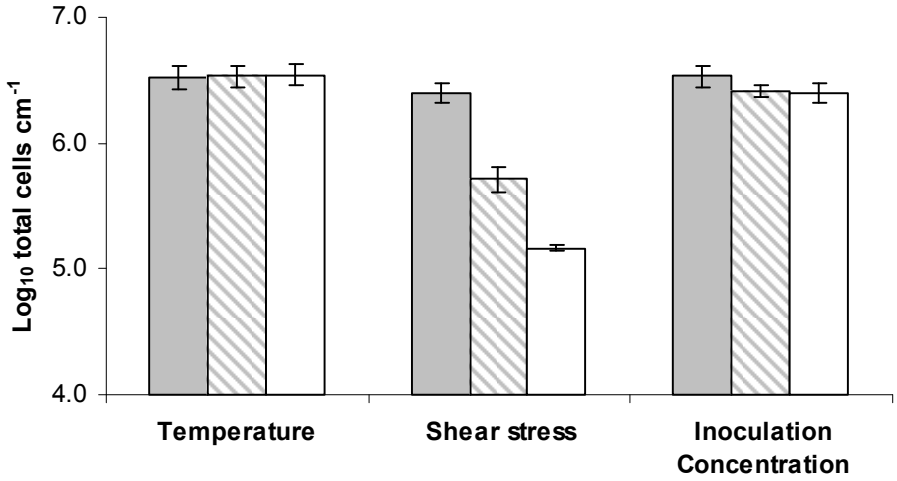


Fig. 3.4. Average of the last three values obtained for different conditions. Bars are ordered in increasing temperature (4, 23 and 37 °C), shear stress (0, 60 and 120 rpm) and inoculation concentration (5.8, 6.4 and 6.8 $\log_{10}$ CFU ml^{-1}). Error bars represent standard deviation.

Although it is known that *H. pylori* expresses outer-membrane-bound adhesins such as BabA, which are capable of interacting with the blood-group antigen $Lewis^b$ on gastric epithelial cells (99), this work shows that it lacks the necessary apparatus to strongly bind to abiotic surfaces when high shear forces are present. This behaviour appears, however, contrary to the one exhibited by most bacteria. Percival *et al.* (190) showed that viable and

total cell counts of an heterotrophic consortium attached on slides of SS304 were higher at flow rates of 0.96 and 1.75 m s^{-1}, compared to a flow rate of 0.32 m s^{-1}. It is also known that *Escherichia coli* can, under certain conditions, adhere more strongly to surfaces with increasing fluid velocity, due to the action of the flagella (150) or of a lectin-like adhesin (235). *H. pylori* should therefore be looked for in water storage reservoirs or well surfaces, where it is likely that the low shear force and high residence time promotes the attachment of the bacterium to surfaces, and consequently associate with biofilms. In fact, one study has found a positive relationship between *H. pylori* infection and well water (107), and others report the detection of the pathogen in these type of systems using molecular methods (8, 95, 123). Although one of the reasons pointed out was the lack of chemical treatment of the water, this work shows that it can also be related to the increased ability of the bacteria to integrate in biofilms under these conditions.

Contrary to our expectations, there was no observable effect of temperature on the adhesion of *H. pylori*. It has been described that the bacterium remains culturable for much longer in water at low temperatures (22 days at 4°C) rather than higher temperatures (6-10 hours at 23°C) (1, 219). It has also been proposed that the attachment and persistence of *H. pylori* on surfaces/biofilms was a phenomenon requiring the organism to be in a viable state (141). If this is the case, and because the length of our experiment was 7 days, this work also suggests that the bacterium is in a viable but non-culturable state for the higher temperatures.

The data obtained about the influence of inoculum concentration is in agreement with work presented by Cerca *et al.* (128), where it is stated that, for each condition, there is a maximum number of bacterial cells that can adhere to a surface, regardless of the initial cellular concentration used. This implies that concentrations of 6.5×10^{5} CFU ml^{-1}, and perhaps even lower, will have the same effect in terms of bacterial attachment, as long as sufficient contact time between the two is established.

3.3.3 Influence of substrata in the adhesion of *H. pylori*

Contrary to what was observed for the adhesion at different conditions, after two hours attachment to certain materials (namely copper, PVC and glass) was found to be lower

than 5 $\log_{10}$ total cells cm^{-2}. After 48 hours, however, all different types of substrata have been colonized by approx. 6.5 $\log_{10}$ total cells cm^{-2} of *H. pylori* (Fig. 3.5).

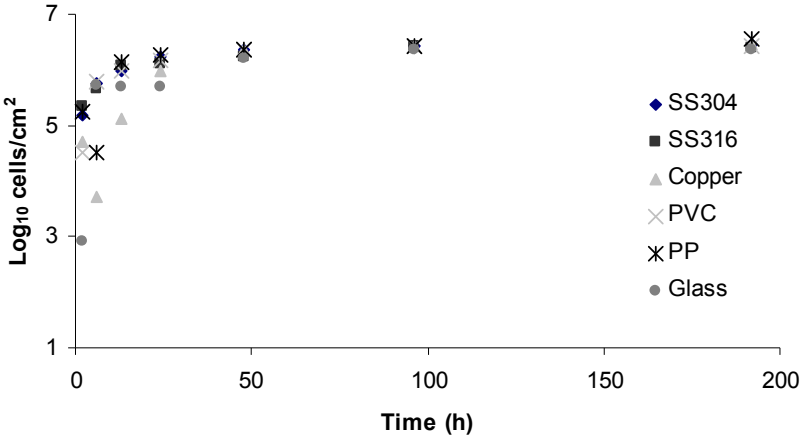


Fig. 3.5. Influence of different substrata on the adhesion of *H. pylori* with time.

It can also be observed from the previous figure that between 48 and 192h, the total number of cells remained nearly constant for all materials. As for the previous section, the system was considered to be in steady-state and the average of these three points was calculated. Copper and PP were found to contain the largest numbers of adhered *H. pylori*, whereas glass was found to have the lowest (Fig. 3.6). The degree of bacterial adhesion to plumbing material substrata after the steady state was reached was found to follow the sequence PP > copper > SS304 > PVC > SS316 > glass (control). However, the student T-test showed no statistically significant differences between the different pairs of materials ($P>0.05$ for all cases). The small differences obtained (2.5% difference between the most extreme case, PP and glass; $P=0.16$) show that, up to 192h, the adhesion of *H. pylori* is not very dependent on the type of substratum used in terms of total cell counts. In previous studies, material composition was a much more important factor in the attachment of pathogens (6, 7), and this work also shows that, after 2 months of exposure, substrata influence is much more noticeable (see Section 3.3.5).

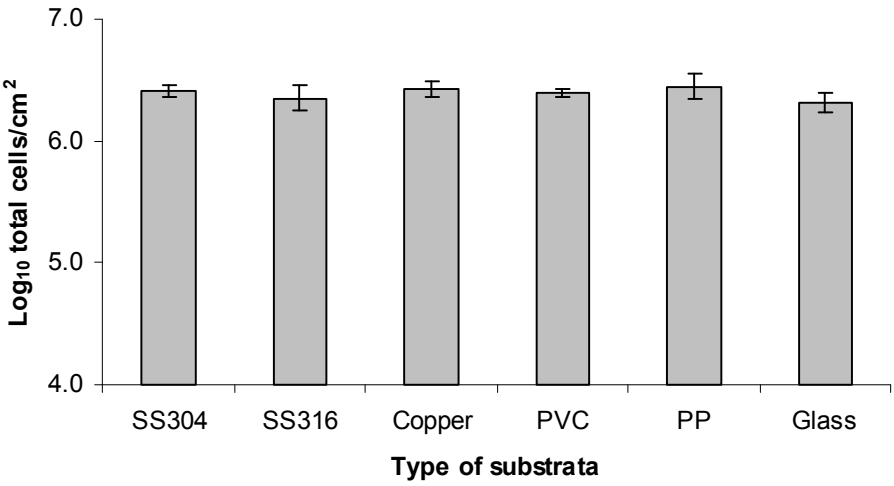


Fig. 3.6. Average of the last three values obtained for different types of substrata. Error bars represent standard deviation.

3.3.4 Relationship between culturability, adhesion, and viability staining

To verify whether any relationship existed between the culturability status of the bacteria and ability to attach to different substrata, the total and cultivable numbers of the bacteria in the planktonic phase were also followed (Fig. 3.7). Culturable cells were below detection after 13 hours (limit of detection, 10 CFU/ml), but DAPI total counts were nearly constant for the time span of the experiment. The levels of total plaktonic bacteria did not decrease with time which confirms the results obtained by Shahamat *et al*. (219). It was also attempted to quantify the number of CFU attached to the coupons, but the methods tested to remove the cells (sonication, scraping and vortexing with glass beads) were only able to detect the bacteria at the two hours exposure time and at very low levels (up to 500 CFU/cm^2) even though the efficiency of removal, as assessed by DAPI total counts before and after the detachment method, ranged between 60-70% for the sonication an vortexing methods. There was little impact of the material on the numbers of cultivable attached bacteria, but this could be due to the lack of a suitable and sensitive method for the recovery of attached bacteria. In an earlier study, Rogers *et al*. (204) highlighted the

importance of this parameter by showing that numbers of *Legionella pneumophila* ranged from 0 CFU/cm^2 for copper to 2,132 CFU/cm^2 for PVC at 20 °C and under shear stress.

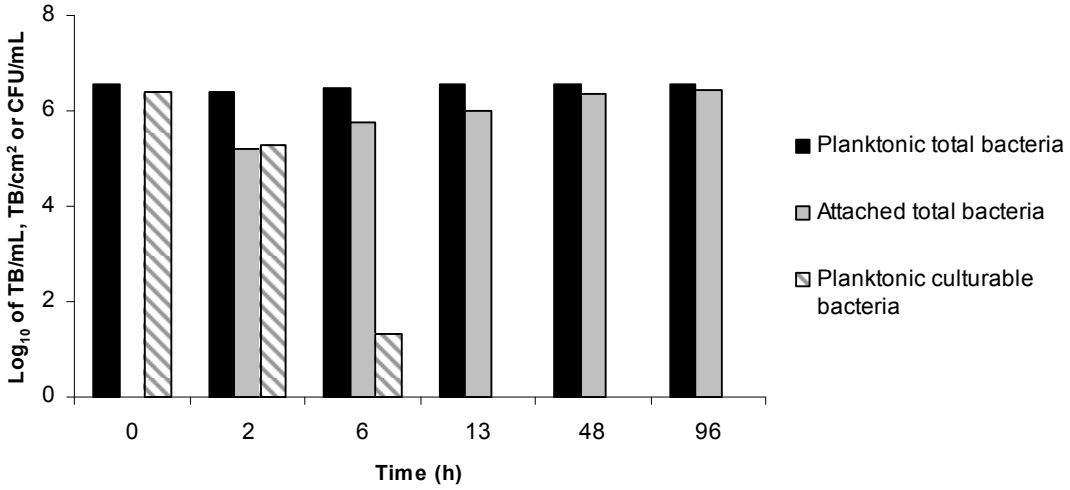


Fig. 3.7. Total number of *H. pylori* cells in the planktonic phase and attached to SS304 substratum and number of viable planktonic cells. For *H. pylori*, attachment of the bacteria could be observed mostly before the loss of culturability.

Syto9/PI analysis of *H. pylori* attached to surfaces showed the large majority of cells starting to take up PI after 48 hours, which is regarded as the beginning of damage occurring to cell membranes. Complete PI uptake was clearly observed in all cells at the end of only one week (Fig. 3.8), which, according to Boulos et al. (56), implies seriously compromised membrane integrity and is therefore indicative of cell death. Although originally it was thought that PI uptake was independent of cell shape, closer observation suggested that coccoid bacteria took longer to stain completely or partially red. This indicates that the coccoid form constitutes a survival strategy in adverse environmental conditions which is in agreement with some recently published studies (39, 213).

Considering the PI uptake as a measure of viability, cells of *H. pylori* enter the viable but nonculturable state after 2 hours, and some cells remain viable for at least 48 hours. After that period of time, this method indicates that attached cells are in a nonviable condition, and therefore unable to cause a public health threat.

Adams *et al*. and Cole *et al*. also used Syto9/PI staining to evaluate the viability of *H. pylori* (1, 41). Cole *et al*. indicated that Brain Heart Infusion Broth (BHIB)-exposed *H. pylori* cells of an actively growing biofilm (3 to 4 days old) were all alive, and that even at day 5, many coccoid cells fluoresced green. The extended resistence to the uptake of PI in this experiment is expected, as culturability of *H. pylori* also increases when it is exposed to BHIB instead of water. Adams *et al*., however, used this method to evaluate viability of planktonic *H. pylori* in a natural freshwater environment. Their results also indicate that the pathogen keeps membrane integrity for at least 100 hours, which surprisingly implies that the pathogen loses viability at a faster rate when attached to surfaces.

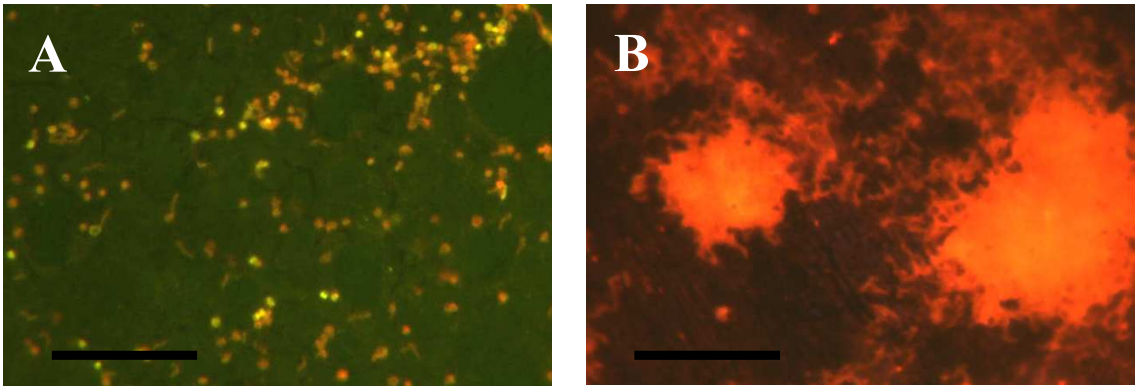


Fig. 3.8. Epifluorescence microscopy images of Syto9/PI stained *H. pylori* on SS304 after 48 hours (a), and of an agglomerate of *H. pylori* cells in copper after 192 hours (b). Bars represent 10 μm.

The relationship between the culturability status of the bacteria and ability to attach to SS304 was studied to verify whether the cell suffers some type of physiological/morphological modification when entering the non-cultivable state that prevents it from attaching. The attachment levels of *H. pylori* to SS304 increased up to 48 hours, regardless of the loss of culturability after only six hours. Although it could be argued that the lower attachment rate after the six hours could be due to viable bacteria under the limit of detection, it is much more likely that the attachment is related to the free area available on the surface. In fact, this logarithmic type curve is typical in studies assessing the attachment of the bacteria to surfaces either for heterotrophic consortia or

monospecies experiments (155, 204). It is therefore unlikely that the rate of attachment to surfaces is related with the culturability state of the bacterium. However, when compared to the results obtained by Syto9/PI staining, it is interesting to observe that attachment stabilizes at the same time cells start to uptake PI.

3.3.5 Morphology and aggregation of *H. pylori* in sessile and planktonic phase

The proportion of coccoid bacteria in relation to the other morphological forms varied clearly with the substrata used for the adhesion. Whereas for polymeric materials, such as PP and PVC, coccoid or U-shaped morphology represented 80%-90% of the total number of cells after 48 hours of exposure, and approx. 95% after 192 hours, for copper it never exceeded 50% even after 192h. For the remaining materials, glass, SS316 and SS304 the proportion of coccoid cells after 192h ranged between 70-85%. The detection of spiral morphology in the planktonic phase occurred until the end of the experiment, but most of the bacteria (approx. 90%) assumed the coccoid morphology after only 48h. Because spiral shape is usually associated with a more active form of the cell (4), the maintenance for longer of spiral morphology of water-stressed *H. pylori* when adhered to copper surfaces can imply that copper provides some sort of protective microenvironment or essential nutrient to the pathogen. Indeed, *H. pylori* is one of the few bacteria that has been shown to possess a copper transporter system (68), which could suggest a higher tolerance of the bacterium for this heavy metal. However, Syto9/PI analysis indicated that cell membrane damage appeared to occur more quickly at the spiral-shaped cells. Taking into account these results, the spiral shape maintenance of *H. pylori* in copper is possibly the result of a fast, biocidal effect of the metal upon the pathogen, killing the cell before it has time to undergo shape modification. The coccoid form has also been recently considered to constitute a survival strategy in adverse environmental conditions (39, 213). Other waterborne pathogens are also known to have adverse reactions to copper. For instance, using copper plumbing has been considered to reduce the risk of *L. pneumophila* transmission through water pipes (204), and the survival of *E. coli* O157:H7 in water has been reported to decrease by elevated copper concentrations (5).

Observations from both SEM and EF show that *H. pylori* is able to form aggregates on the surface of the different substrata. The structure and abundance of these aggregates also varied with the support material but appeared not to be affected by the other conditions tested. For instance, copper promoted the accumulation of large numbers of bacteria (more than 50 cells), either in a monolayer or in several layers after 96h (Fig. 3.9a). Agglomerates of a medium size (10-50 cells) could occasionally be found on PP and PVC after the same time, but were mostly constituted by cocci or U-shaped bacteria and disposed in a monolayer (Fig. 3.9d). Large aggregates were not detected before 96h for any material and were non-existent in the planktonic phase during the time course of the experiment. Smaller aggregates of less than 10 cells were ubiquitous in all materials and could be sparsely found in the planktonic phase after only two hours (Fig. 3.9b,c). When aggregated between them, coccoid forms appeared to be able to change their shape in order to accommodate other cells, creating a still unreported pentagon-like morphology (Fig. 3.9d).

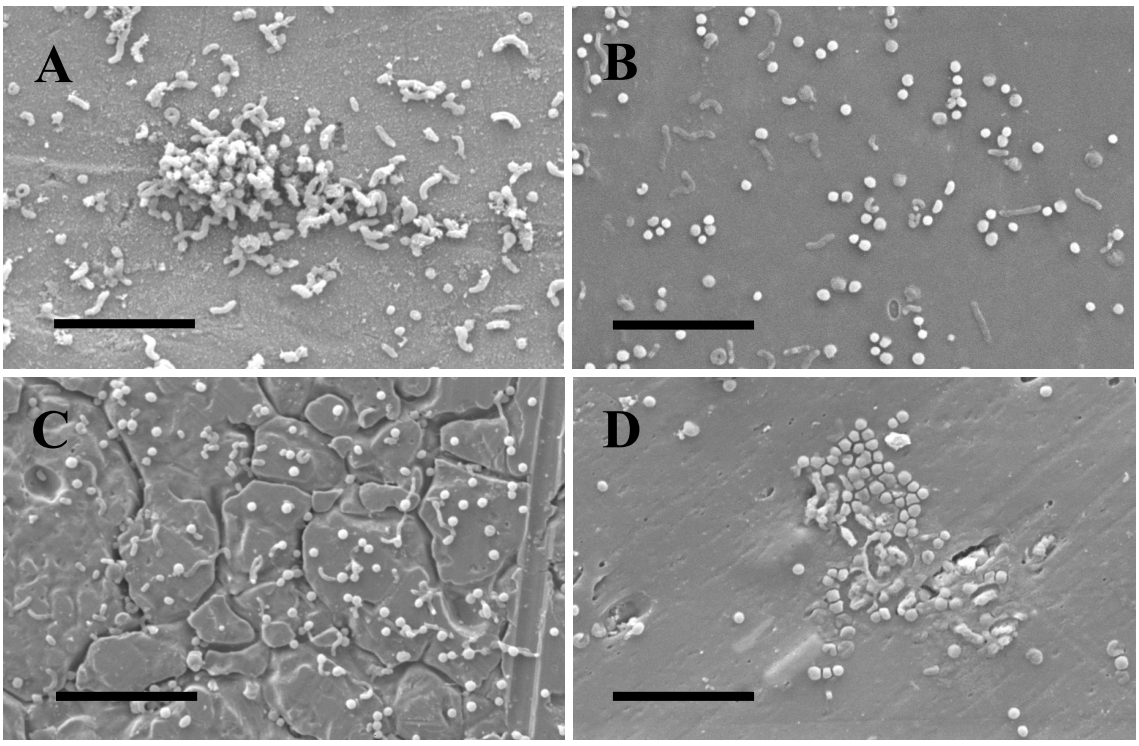


Fig. 3.9. SEM images obtained for *H. pylori* adhesion after 192 h on copper (a), glass (b) SS304 (c) and PVC (d). Bars represent 10 μm.

After two months, on the copper and SS304 substrata, most of the bacteria were arranged in the form of large aggregates, indicating that aggregation of *H. pylori* when attached to water-exposed surfaces tends to occur after longer periods of exposure. However, there was no evidence of EPS being produced on any of the substrata. On copper, two distinct areas could be observed: a darker area where *H. pylori* tended to agglomerate, form well-defined 3-D structures and be in higher numbers (Fig. 3.10), and a brighter area where *H. pylori* tended to be in lower numbers and where the 3-D structures appeared to have collapsed. Aggregates in SS304 also resembled this latter 3-D collapsed structure.

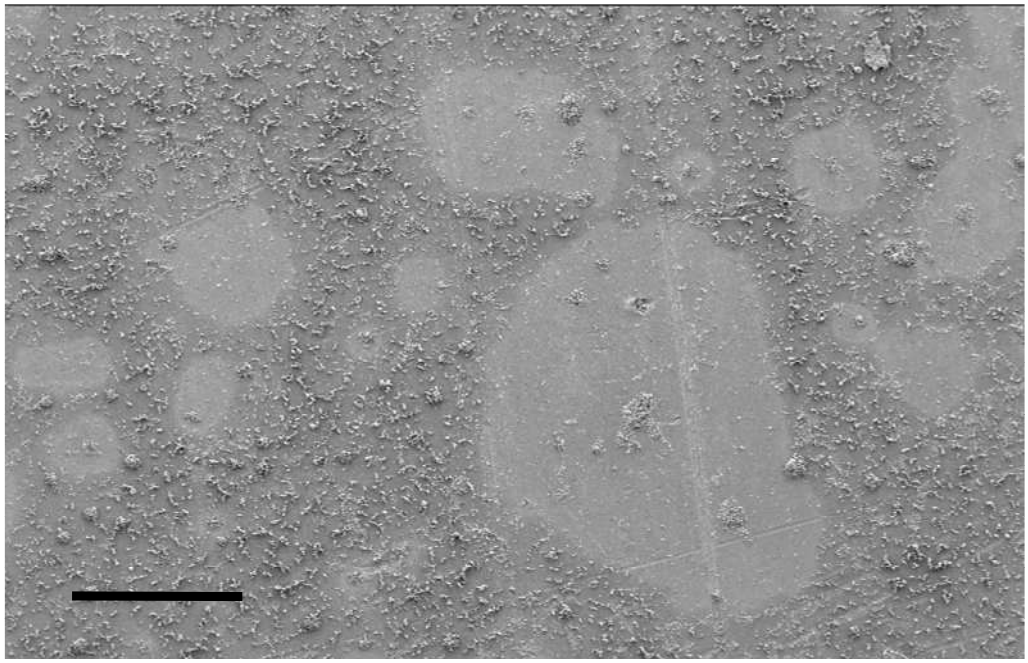


Fig. 3.10 SEM image of a 2 month *H. pylori*-exposed copper surface, where bright and dark areas are clearly visible. Bar represents 200 μm.

X-ray microanalysis of the copper coupons, showed that in the brighter area a larger oxygen peak could be detected, indicating that this area corresponded to a more oxidized area of the copper surfaces (Fig. 3.11).

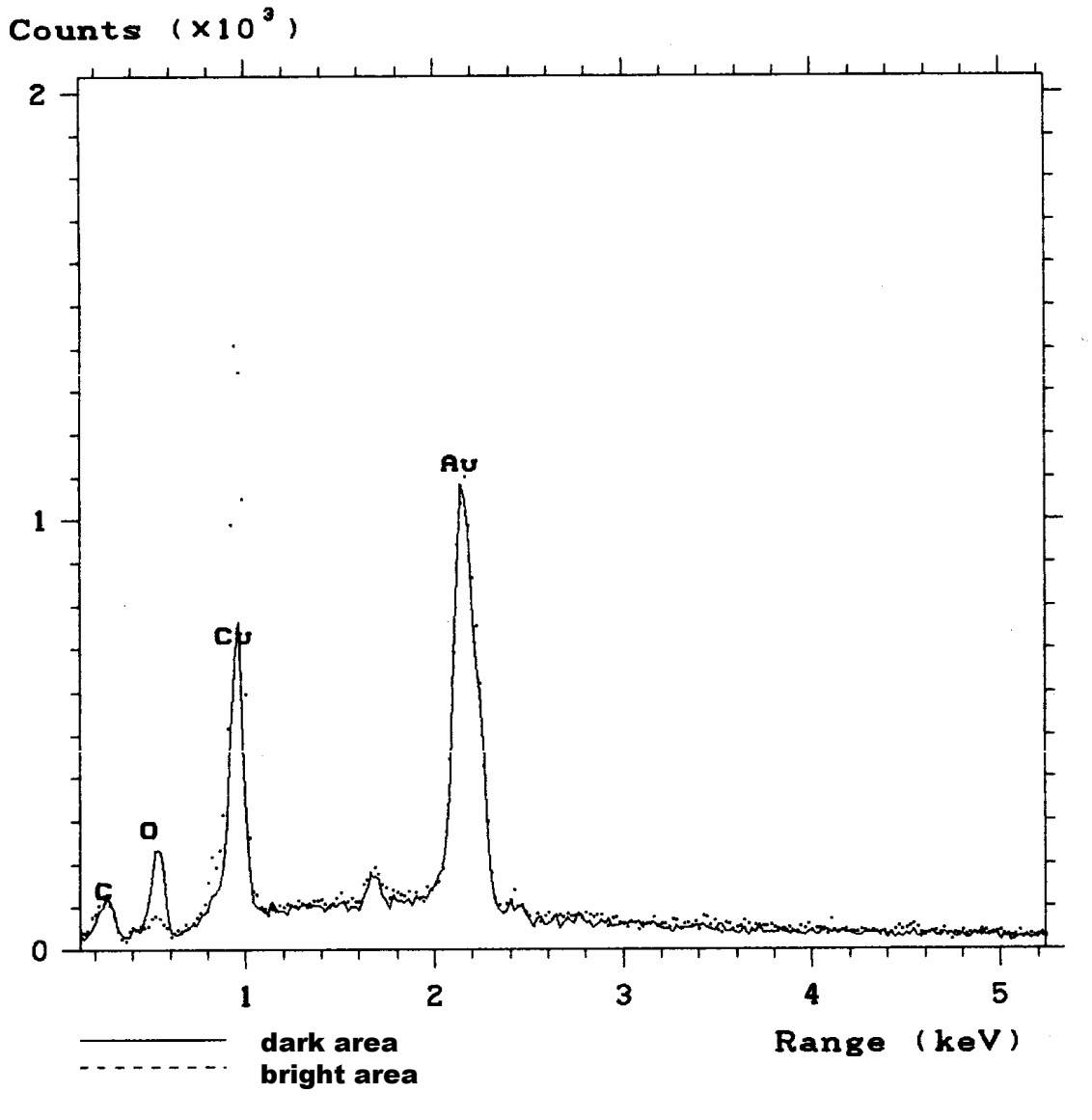


Fig. 3.11. X-ray microanalysis of a dark and bright area of a copper coupon exposed for two months in a *H. pylori* suspension.

Cole *et al.* (41) reported that *H. pylori* was only able to form biofilms at the air-liquid interface, and that the 3-D structures could be observed after 3 to 5 days. This work proves that *H. pylori* is also able to form 3-D structures on the surface of metallic-surfaces (mainly copper) when these are completely immersed in water. Even though the formation of the first structures occurred after 8 days, it is not clear whether this difference is

explained by the absence of an air-liquid phase, the different liquid phase used (BHIB in Cole *et al*. and distilled water in our case), the different type of substrata used, or even more likely, a combination of two or all of the three factors. The fact that on glass, the same substrata used by Cole *et al*., only small aggregates could be observed up to eight days does indicate that biofilm formation in water and BHIB appears to follow the same rules. It would be interesting to observe coupons of glass and the plastic-based materials to observe if 3-D structures would also be formed after longer periods of time, especially because, as it has been already mentioned, formation of three-dimensional structures usually creates microenvironments where the bacteria can have better chances of survival. Because it has been shown that in some cases, simple *in vitro* studies, utilizing only one or a few species, are poor models for adhesion and colonization in natural environments (257), studies involving natural heterotrophic microbial flora should be performed in the sequence of this work.

During the time course of the experiment large aggregates were only found adhered to materials, especially copper, but it has already been observed that *H. pylori* would aggregate in the planktonic phase after longer periods (219). This was also observed in our study at times of water exposure of more than one month.

3.3.6 Contact angles relationship

This part of the work was performed to verify whether the thermodynamic theory of adhesion and degree of hydrophobicity correlated well with the experimental values obtained for the attachment of the bacteria. As for the substrata influence experiment, results show that *H. pylori* is able to adhere to all materials, with numbers of adhered bacteria to glass being the lowest obtained (Fig. 3.12). One way ANOVA analysis followed by a LSD *post hoc* test has shown that differences in the adhesion between the different materials are not statistically significant ($P>0.05$). The only exception was for the comparison between SS304 and glass ($P=0.036$).

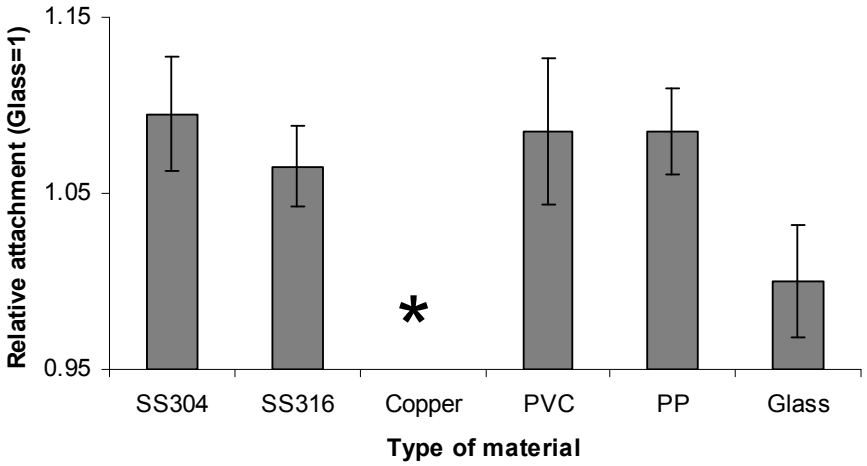


Fig. 3.12. Comparison between the adhesion values obtained by the DAPI method for the materials tested. Values were obtained by dividing the total counts of *H. pylori* attached to the different materials by the total counts of *H. pylori* attached to glass.* Cell counts on copper were not considered in this assay (see Section 3.3.1).

For the determination of contact angles between the chosen liquids and *H. pylori*, the time needed for the contact angle between water and the lawn to reach stable values (or plateau) was determined (Fig. 3.13). This state of drying indicates that only bound water is present on the surface (245). For this experiment, the plateau was reached after 2.5 hours, which is in agreement with existing bibliography, where microbial lawns are also left to dry in a Petri-dish with glycerol (220).

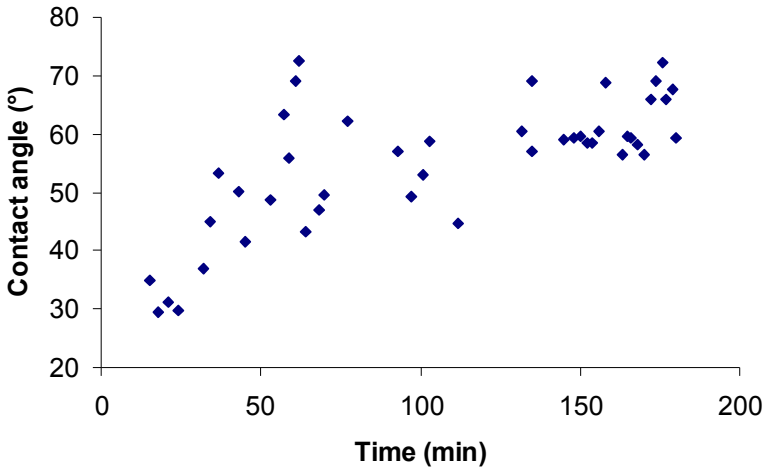


Fig. 3.13. Contact angle between *H. pylori* microbial lawn and water varying with time.

In terms of surface characteristics and observing Table 3.2, it appears that most materials were hydrophobic, based on the threshold of 60° of the contact angle between the surface and water described in Reid *et al.* (200). Based on the same value, *H. pylori* cells can also be considered slightly hydrophobic. However, other authors have indicated different threshold values, ranging from 50 to 65° (249, 253). Previous studies reporting the water contact angle with *H. pylori* have indicated much lower values, such as an average of 8.9° for strains isolated from children with gastritis (131), or approx. 40° for a 15-day old plate culture of the laboratory strain 88-23 (61). Although large disparities have been already found between water contact angles of microorganisms of the same species but different strains (245), in this case the differences might be better explained by the different physiological status of the bacterium when the contact angle was performed. Increasing hydrophobicity of the *H. pylori* surface has been correlated with increased time of incubation of the bacterium on plates (61).

Table 3.2. Values of contact angles measured with water (θ_w), formamide (θ_f) and 1-bromonaphthalene (θ_b) for lawns of *H. pylori* NCTC 11637 and different test surfaces. Results for the test surfaces were obtained before and after preparation.

Test surface		θ_w (± SD)	θ_f (± SD)	θ_b (± SD)
Before treatment	SS304	72.2 (± 7.5)	60.5 (± 6.2)	21.2 (± 3.1)
	SS316	73.4 (± 5.1)	62.6 (± 3.4)	22.2 (± 3.5)
	Copper	90.6 (± 2.8)	74.1 (± 3.5)	22.0 (± 2.5)
	PVC	79.0 (± 5.8)	50.6 (± 3.7)	18.7 (± 3.2)
	PP	n. p.	n. p.	n. p.
	Glass	54.4 (± 2.2)	24.9 (± 5.4)	34.9 (± 4.0)
After treatment	SS304	76.3 (± 6.9)	67.0 (± 9.3)	48.4 (± 6.3)
	SS316	63.6 (± 10.8)	60.7 (± 7.4)	32.9 (± 4.2)
	Copper	112.1 (± 2.7)	67.0 (± 9.3)	48.8 (± 9.4)
	PVC	86.0 (± 6.4)	68.6 (± 4.8)	25.0 (± 3.4)
	PP	98.1 (± 5.7)	81.0 (± 5.5)	44.5 (± 6.1)
	Glass	38.8 (± 5.1)	34.3 (± 6.7)	38.1 (± 7.2)
H. pylori NCTC 11637		62.4 (± 4.7)	72.5 (± 6.8)	62.5 (± 4.6)

n.p. – not performed

For microbial species, the 1-bromonaphthalene contact angle usually varies less than water and formamide contact angles and ranges between 22-61° (245). The value for the *H. pylory* and 1-bromonaphthalene contact angle is slightly above this interval, denoting the ability of the cell surface to exert Lifshitz – van der Waals forces (248).

The percentage of difference between contact angles from surfaces without preparation to surfaces with preparation ranged from 5 to more than 100%, depending on the liquid used. Largest deviations were observed in the measurements with 1-bromonaphthalene, where increases of more than 50% occurred for nearly all materials.

In Table 3.3, the values of the degree of hydrophobicity for the materials and *H. pylori* cell surfaces (ΔG_{sws}) are presented. As referred in the Materials and Methods, ΔG_{sws} was determined with the values $\gamma+$, $\gamma-$ (electron-acceptor and electron-donor parameters of acid–base component of the surface tension) and γ^{LW} (the Lifshitz van der Waals

component of surface tension), which were calculated with the contact angles of water, formamide and 1-bromonaphthalene as in Henriques *et al.* (101).

Table 3.3. Values of surface tension components (γ^+, γ^-, γ^{LW}) and degree of hydrophobicity (ΔG_{sws}) of the test surfaces after preparation in mJ/m^2.

Test surface	γ^+	γ^-	γ^{LW}	ΔG_{sws}
SS304	0.00	14.37	30.74	-26.95
SS316	0.22	27.21	37.58	-1.21
Copper	1.98	4.08	30.55	-45.61
PVC	0.35	5.95	40.32	-52.19
PP	0.58	2.81	32.60	-60.05
Glass	0.98	40.11	35.44	17.55
H. pylori	0.14	41.27	23.72	25.61

After the calculation of surface free energy components and parameters, all but one of the test surfaces studied showed negative values of ΔG_{sws}. As expected, glass exhibited a positive ΔG_{sws} – and is therefore hydrophilic. Interestingly, the ΔG_{sws} value for *H. pylori* shows a very hydrophilic surface. The bacterium also exhibited a high value for the electro-donor parameter, which is typical for Gram-negative bacterial cells and is correlated with having low nitrogen, oxygen and phosphorous content on their cell surfaces (220).

The exact role and importance of hydrophobicity and cell surface energy in the adhesion of microorganisms to surfaces remains unclear (227). For instance, Briandet *et al.* (25) and Chavant *et al.* (78) have both concluded that hydrophobicity is involved in *Listeria monocytogenes* adhesion to stainless steel, whereas capsule production, but not hydrophobic interactions, was associated in the attachment of *Escherichia coli* O157:H7 to food surfaces (85).

A comparison between the experimental values obtained by the adhesion tests and the theoretical values based on the LWAB theory demonstrated that *H. pylori* adheres better to the more hydrophobic surfaces, regardless of having a positive value for the degree of

hydrophobicity. The theoretical prediction of the extent of adhesion can be better explained after the calculation of the free energies of adhesion (Table 3.4). In fact, the total counts on the surfaces (from highest to lowest) varied in the following order SS304>PP>PVC>SS316>Glass, while the free energy of adhesion (from most to less favorable) was PP>PVC>SS304>SS316>Glass. Therefore, the only exception for a perfect correlation was SS304, where the number of cells attached was greater than to PVC and PP, despite the fact that, according to the free energy of adhesion values, SS304 is less likely to support adhesion than these two materials. It is important to bear in mind, however, that differences in the adhesion between nearly all materials are not statistically significant, and as such, conclusions about the correlation have to be interpreted with caution.

Table 3.4. Values of the free energy of adhesion (ΔG_{adh}) and its apolar (ΔG_{slm}^{LW}) and polar (ΔG_{slm}^{AB}) components for the system surface-water-*H. pylori* in mJ/m^2.

Test surface	ΔG_{slm}^{LW}	ΔG_{slm}^{AB}	ΔG_{adh}
SS304	-0.352	2.108	1.756
SS316	-0.588	14.149	13.561
Copper	-0.345	-18.320	-18.665
PVC	-0.677	-12.156	-12.833
PP	-0.419	-19.758	-20.177
Glass	-0.517	23.162	22.645

H. pylori NCTC 11637 adhered in higher numbers to the more hydrophobic surfaces. An inverse correlation was observed between ΔG_{sws} and attachment numbers, with SS304 being the only exception. Therefore, and even though other factors might also be of importance for this process, the degree of hydrophobicity appears to play a role in the adhesion of *H. pylori* to pipe surfaces. Equally, ΔG_{adh} predicted accurately the adhesion extent but for SS304.

3.4 Conclusions

This study represents, to the best of the author's knowledge, the first report on the effect of different conditions on the attachment of *H. pylori* to a pipe material. It was observed that shear stress was the single most important factor to influence *H. pylori* adhesion. This will hopefully lead researchers to perform a more rationale selection of locations, such as wells or reservoirs, to perform molecular or plate culture analysis for the detection of *H. pylori*.

This work has also highlighted the ability of *H. pylori* to adhere to different plumbing materials, which would support the theory of a drinking water route for the transmission of the pathogen. Copper surfaces are especially suitable for the maintenance of the bacteria in the spiral form, as well as for the formation of a larger number of agglomerates in the surface. However, the physiological state of *H. pylori* under those conditions, as determined by Syto9/PI viability and culture medium experiments, has indicated that most of the bacteria attached have a compromised membrane and are in a non-cultivable state. It would be expected that the formation of aggregates and maintenance of a spiral morphology required the cell to be in a viable form, but contact angles data suggest that these events can occur with inactive forms of cells and be solely explained by physical processes invoked, for example, in the thermodynamic or DLVO theory of cell attachment. This is demonstrated by the fact that the thermodynamic theory of adhesion and degree of hydrophobicity correlated well with the values obtained for the attachment of the bacteria.

The apparent inability of *H. pylori* to remain viable in monospecies biofilms, implies that co-aggregation with other bacteria might be essential for its survival. Further research in this area needs therefore to be performed.

4 *H. pylori* survival and culturability in water

Arguably, the main reasons for water not to be widely considered as an extra-gastric reservoir for *H. pylori* relates to the low survival time of the pathogen in this environment and the failure to isolate it from water supplies. This Chapter assesses the importance of different factors in the recovery of the pathogen from water, with the purpose of improving the current understanding of its behaviour in those environments and hence clarify the mode of transmission debate. For that, different incubation techniques are tested for an improved isolation of the bacteria from water. The development and evaluation of a new culture medium that was specifically designed to recover the bacteria from low-nutrient environments is described, and the suitability of a battery of antibiotics, referred in the literature for the selective isolation of *H. pylori* from water, for this new medium was also tested.

4.1 Introduction

Shahamat *et al.* (219) determined that *H. pylori* total cell counts in water would not decrease for very long periods (2 years at 4 °C) using the acridine orange staining procedure, an experiment that was repeated in our laboratory using DAPI (unpublished data). Although AO and DAPI use is associated only with total bacterial counts and is not able to discriminate between live or dead cells, the fact that the bacteria could still keep the cell wall, the morphology and DNA intact for so long raises questions whether they are in a viable state or not. However, viability is intimately associated with culturability, and *H. pylori* culturability in water usually ends after a short time (1, 219). Furthermore, demonstration of the existence of viable *H. pylori* either in the planktonic phase or in biofilms associated with drinking water using plate-spreading techniques has yet to be accomplished. Excluding the fact that only very recently have these surveys started to be performed on a large scale (52), only two other distinct explanations can be advanced for this apparent failure: either *H. pylori* is in fact not able to survive in potable water systems, or some of the recovery technique parameters, such as the media composition and the incubation atmosphere, are not adequate for replication and colony formation when the pathogen is water-stressed.

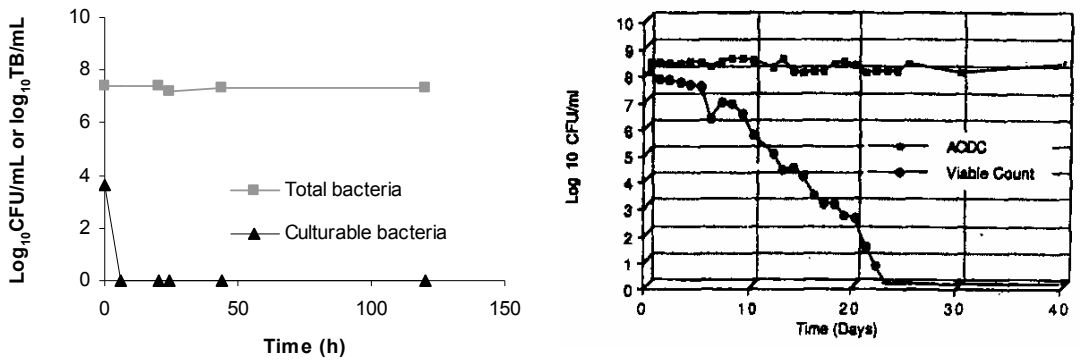


Fig. 4.1. Total and culturable cell counts of *H. pylori* at different water exposure times at 23 °C (left) and 4 °C (right). Right graphic is a reproduction from Shahamat *et al.* (219).

4.1.1 Pathogen survival in drinking water and the viable but non-culturable (VBNC) state

Pathogens will have a variable persistence upon discharge into a water source, depending on the ability of the microorganism to cope with the environmental stress caused by this hostile microenvironment. Some of the most common waterborne bacterial pathogens are described in Table 4.1, together with culturability times in water (*i. e.* time the bacteria will remain in a culturable state when exposed to water) obtained at different temperatures. As can be observed, water temperature strongly influences the culturability of many pathogens, with lower temperatures generally allowing longer culturability times. Also, *H. pylori* has the lowest culturability time of all the microorganisms included in the table, which partially accounts for the reason why the scientific community has serious questions about the possibility of it being a waterborne pathogen. Nevertheless, culturability times between *H. pylori* and *C. jejuni* are of the same order of magnitude, suggesting that the close taxonomical relationship between these bacteria is reflected in the way they both similarly withstand the stress caused by water. Waterborne outbreaks of gastroenteritis due to *C. jejuni* transmitted through water supplies are commonly reported in the literature (e.g. 71, 229).

Table 4.1. Comparison of the culturability times in water, described for different temperatures, between different waterborne pathogens and *H. pylori*.

Microorganism	Culturability	T (°C)	References
H. pylori	≈ 6 hours	23	Adams, B. *et al.* (1)
	24 days	4	Shahamat, M. *et al.* (219)
Yersinia enterocolitica	10 days	30	Chao, W.-L. *et al.* (36)
L. pneumophila	36 to 42 days	42	Ohno, A. *et al.*(175)
	>60 days	25	
E. coli O157	49 to 84 days	25	Wang, G. and M. Doyle (255)
	91 days	8	
S. typhimurium	>45 days	20-30	Santo Domingo, J. *et al.* (216)
C. jejuni	43 hours	22	Buswell, C. *et al.* (33)
	29 to 36 days	4	Cools, I. *et al.* (46)

In addition to the very low culturability time in unchlorinated water, the survival ability of *H. pylori* in treated water systems was questioned, with the suggestion that this microorganism was very sensitive to the chlorine treatments used in water treatment plants (105). However, Baker and Hegarty argued later that *H. pylori* could tolerate disinfectants better than the classical faecal indicator, *E. coli* (9). Consequently, disinfected water could be free of coliforms and classed as safe, but might still contain potentially infectious *H. pylori* that could therefore be transmitted by a waterborne route.

While culturability using standard plating techniques has been for a long time the gold-standard to assess the survival of a microorganism in a given environment, the development of new methods to evaluate the same parameter (24) has questioned the ability of a single cell to divide in artificial nutrient media as a suitable indication of the cell's life and death. The notion of viable but nonculturable (VBNC) has then arisen to describe organisms that are not culturable at a given time or condition, but may revert to a state of culturability later or under different circumstances (174). Resuscitation techniques have therefore been developed for several microorganisms to alleviate this state, again enabling culturability (e.g. 223, 265). In particular, it has been shown for *C. jejuni* that plate counts underestimated the true viable count by up to 23-fold (22).

4.1.2 Media development and incubation strategies for the recovery of *H. pylori* from water

Some of the most successful approaches to grow in culture medium yet uncultured or environmentally-stressed microorganisms are due to use of growth conditions which closely mimic the chemical composition of natural environments (44). In terms of water heterotrophic plate count analysis, a major breakthrough was achieved in 1985, when Reasoner and Geldreich reported the development of the R2A medium (199). In the original study, the new medium yielded significantly higher bacterial counts than did plate count agar, the recommended medium at the time, for assessing the heterotrophic consortia present in water. R2A was developed on the basis that plating medium must provide not only the essential organic substrates necessary for the fastidious microorganisms in the heterotrophic bacterial population, but also provide a dilute nutrient

base that might approach the chemical constituents concentrations in distribution water (69).

Several media have been assessed for the recovery of *H. pylori* from different environments, including infected individuals (79, 194) and cattle and beef samples (233). However, the matrices involved are all nutrient rich. By contrast, for microorganisms to survive in natural waters, they must adapt physiologically to a low nutrient environment. As a result, the traditional recovery media may be too nutrient rich for their optimum culturability: a condition described here as nutrient shock. Therefore, we have tested the hypothesis of nutrient shock as a hampering factor in the recovery of *H. pylori* from water using spread plate procedures.

Another strategy that has been commonly used to recover uncultured microbes from the environment has been the incubation atmosphere. For instance, Stevenson *et al.* reported that members from agricultural soil and from the guts of wood-feeding termites, such as Acidobacteria, were found in higher quantity on isolation plates that had been incubated with 5% CO_2 (232). *Helicobacter* spp. are described as microaerophiles, requiring a low oxygen and balanced carbon dioxide atmosphere to grow. One study has also concluded that hydrogen is an energy-yielding substrate that can facilitate the maintenance of the bacterium (179). However, studies published so far regarding incubation atmospheric conditions concerned commercially available kits and did not make a systematic study about the influence of atmospheric concentrations (87, 247). The availability of microaerophilic cabinets with adjustable atmospheric control allows a more accurate understanding of the effects of the incubation atmosphere throughout the incubation period and select for the best atmospheres needed for different microorganisms to grow. A Modullar Atmosphere Controlled System (MACS) workstation was therefore used in the current study to define the optimal oxygen, carbon dioxide and hydrogen concentrations to recover potentially stressed *H. pylori* from water.

A further major difficulty in attempting to culture *H. pylori* from natural environments is the presence of authoctonous microbiota. These bacteria tend to grow much more rapidly than *H. pylori* and hence possibly mask its presence. With that in mind, Degnan *et al.* have recently developed a plating medium to allow large-scale screening of water samples for

the specific presence of *H. pylori*, using an assortment of antibiotic supplements (52). The medium has been tested for the selectivity of *H. pylori* against both natural and spiked water and, in spite of the heavy mixed microbial load, only *H. pylori* colonies grew during 7 days of incubation at 37 °C.

4.2 Material and methods

4.2.1 Culture maintenance

For studies involving the nutrient shock and incubation atmosphere experiments, *H. pylori* NCTC 11637 was maintained on Columbia Agar (CA) (Oxoid, UK) supplemented with 5% (v/v) defribinated horse blood (Biomérieux, France) in the presence and absence of 1% (v/v) fetal calf serum (Merck, Germany). Plates were incubated at 37 ^{o}C in 2.5 L jars (Oxoid, UK) under microaerophilic conditions created using a CampyGen gas pack (Oxoid, UK) and streaked onto fresh plates every 2 or 3 days. For the remaining studies, *H. pylori* NCTC 11637 was incubated at 37 ^{o}C in a 2.5L GENbox (BioMérieux) under microaerophilic conditions created using a Microaer gas pack (BioMerieux).

For the new medium evaluation 7 additional clinical isolates (three obtained from adults and four obtained from children) were tested. The clinical isolates belong to the strain collection of the National Institute of Health in Lisbon, Portugal. Clinical isolates were stored at -80 ^{o}C upon arrival and subcultured only once before the medium evaluation experiment, in the same conditions as the reference strain.

4.2.2 Nutrient shock influence

Induction of water-stressed H. pylori and subsequent recovery

Cells from 2 day-old cultures were harvested from Columbia Agar supplemented with 5% (v/v) defribinated horse blood plates (hence denominated CBA) and suspended in 10 mL of autoclaved tap water for a concentration of ca. 10^8 CFU per ml to be achieved. This inoculum was then transferred to a sterile 1000 mL bioreactor, also containing autoclaved tap water, to achieve a final concentration of ca. 10^6 CFU/ml. The bioreactor was maintained at room temperature (approx. 24±2°C) and continuously stirred (120 rpm)

using a magnetic bar. Sampling was performed at different times up to 24h. Before serial dilution (1:10) in sterile tap water, samples were vortexed for 10s for homogenization. *H. pylori* was enumerated in triplicate by plating 100 µl of the different dilutions onto the appropriate agar media. Plates were incubated at 37 ºC for 6 days under the same microaerophilic conditions used for culture maintenance.

Media preparation

Three different media, Columbia agar (CA) (Oxoid, UK), *Helicobacter pylori* special peptone agar (HPSPA) and Wilkins-Chalgren agar (WCA) (Oxoid, UK) were compared for recovery of *H. pylori* from water samples. CA and WCA were prepared according to the manufacturer's instructions. HPSPA was prepared as described previously (233), by adding to distilled water: Special Peptone (Oxoid, UK), 10 g/l; yeast extract (Merck, Germany), 5 g/l; beef extract (Merck, Germany), 5 g/l; sodium chloride (Merck, Germany), 5 g/l; pyruvic acid: sodium salt (Sigma, USA), 0.5 g/l and granulated agar (Merck, Germany), 15 g/l. After allowing the three different media to cool down to 55 ºC following the autoclaving process, 5% (v/v) defribinated horse blood was added. For each set of experiments, media were prepared and poured into plates two days before the experiment, and stored at 4 ºC.

To study the effect of nutrient shock on *H. pylori*, the media mentioned above were prepared in half and a quarter strength, by reducing the constituents 2 to 4-fold. The agar concentration was maintained constant. The media are described as half strength and quarter strength. Evaluation of the growth in different media was accomplished by counting colony forming units and measuring colony size.

4.2.3 Incubation atmosphere influence

Using a variable atmosphere workstation (MACS VA500, Don Whitley, Shipley, U.K.) several atmospheres were tested and compared with Campygen gas generation systems. To ensure the necessary humidity in the work station, which was set to 95%, a beaker with approx. 300 mL of water was placed inside it. From preliminary experiments, it was decided that only one sample taken after a relatively short time of exposure to water would

reflect the differences between the two gas generation systems. Therefore, after 10 min. a sample was taken from the bioreactor (as described in the previous section) and cultured on CBA. To provide a better sensitivity in the method, six agar plates were spread for each experiment.

4.2.4 Development of a new low nutrient medium

Because in the nutrient shock experiment, the use of nutrient-rich media for the assessment of water-stressed *H. pylori* proved to have a deleterious effect on the recovery of the pathogen when compared to low nutrient media, the development of a new low nutrient medium was attempted. However, that same experiment reported that diluting the media too much would prevent the pathogen from forming colonies. Based on these results it was attempted to develop a medium that could support *H. pylori* growth, but contain as low a concentration of nutrients as possible. The first step was to identify the medium that supported best growth between the three base media, and then identify the component, or components, which halted the growth of *H. pylori* when that medium was diluted by more than a factor of two.

Comparison between the three base media

To assess which of the three base media yielded higher counts, a similar experiment as the one described in 4.2.2 was performed. Instead of comparing the different nutrient contents of each media, the three base media were compared between themselves. Suitability of the media to provide a base for a future low-nutrient medium was assessed by counting colony forming units and measuring colony sizes. To avoid possible interferences of the culture maintenance medium in the final results (due to *H. pylori* adaptation to that medium while it is being subcultured), three replicate experiments were performed, but for each of them *H. pylori* biomass was either collected from CBA, HPSPA or WCA culture maintenance plates.

Development of a new medium based on HPSPA

Using HPSPA as the reference medium and *H. pylori* NCTC 11637 as the test strain, several sets of experiments were conducted (Table 4.2). In each set, media were prepared by varying the concentration of one of the individual components in half and quarter-strength HPSPA. An initial assessment of the media performance was obtained by plating an appropriate quantity (between 20 and 200 CFU per plate) of water-stressed *H. pylori* on the different media and comparing the results with the number of colonies obtained on a standard HPSPA medium. Mean colony size was also obtained by measuring the sizes of 10 or more colonies of one plate, using plates with similar number of colonies.

Table 4.2. Range of the concentrations of components from the HPSPA media tested for *H. pylori* growth.

Component	Range of concentrations tested[b]	Minimal concentration to support acceptable growth on half-strength HPSPA[a,b]	Minimal concentration to support acceptable growth on quarter-strength HPSPA[a,b]
Special Peptone	1-10	3	n.p.[d]
Yeast extract	1.5-5	1.5	5
Meat extract	1-10	1	10
Sodium pyruvate	0.1-1	0.1	n.p.
Sodium chloride	0-5	2.5	5
Granulated agar	10-15	10[c]	n.p.
Horse blood	1-5	2.5%	n.p.

[a]The medium was considered to support acceptable growth if colony counts recovered in that medium were in the same order of magnitude as the reference media, HPSPA.

[b]In %(v/v) for defribinated horse blood and g/L for every other component.

[c]Concentrations of agar lower than 10 g/L prevented the medium from solidifying properly.

[d]Growth is not supported at acceptable levels even when the highest concentration was tested.

Based on the results obtained and summarized on Table 4.2, several new low nutrient media were designed and subsequently compared with HPSPA. The concentration of the components in HPSPA and the new low nutrient formulations (designated wHPA) can be found in Table 4.3.

Table 4.3. Composition of HPSPA and different wHPA media.

Component (g/L) or %(v/v)	HPSPA	wHPA	wHPA(1)	wHPA(2)
Special Peptone	10	2.5	3	3
Yeast extract	5	4	2.8	2.6
Meat extract	5	1	1.5	1.5
Sodium pyruvate	0.5	0.2	0.1	0.5
Sodium chloride	5	5	5	5
Granulated agar	15	10	10	10
Horse blood	5	2.5	2.5	2.2

4.2.5 Alternative strategies to improve recovery

Other strategies were also tested to attempt recoveries of higher numbers of *H. pylori* from water. In all of the tests, the induction of water-stressed *H. pylori* and subsequent recovery was performed as in Section 4.2.2. The first strategy involved filtering 0.5 mL of the *H. pylori* suspension through 47 mm diam., and either 0.1, 0.2 or 0.45 μm pore size, membranes (in triplicate) (Pall Gellman, U.S.A.). During incubation, these membranes were transfered onto fresh HPSPA plates every 7 days, to recover possible late-growing *H. pylori* colonies. Total incubation time was nearly 2 months. The second strategy involved replacing the NaCl component by KCl (Sigma, U.S.A.) in HPSPA medium (in quadruplicate). Plating/filtering occurred after exposure of *H. pylori* to water for 1.5-2 hours.

4.2.6 New medium evaluation before and after inclusion of antibiotics

In all of the tests, the induction of water-stressed *H. pylori* and subsequent recovery was performed as in Section 4.2.2. Sampling was performed at a single time (after 2 hours) for all strains, by plating 100 µl in three to twelve plates of HPSPA or wHPA. For NCTC 11637, one experiment was also carried out with time (up to 10 hours), with four plates of each medium.

For the evaluation of described antibiotics in the new medium, both HPSPA and wHPA were prepared by the standard method, with 3,500 U of polymyxin B, 7.5 mg of amphotericin B, 10 mg of vancomycin, 5 mg of trimethoprim, 5 mg of cefsulodin per liter (all from Sigma) being added aseptically after tempering to 50°C. These media were compared with the antibiotic-free media using the procedure described above, with NCTC 11637 as the test strain.

CBA without antibiotics and HPSPA with antibiotics were also tested against microorganisms that have been isolated from drinking water biofilms, and identified using either API (ID32 GN) or growth on selective media. Culture maintenance, colony morphology and gram staining characteristics of the isolates are described in Chaves (37). For all microorganisms, 3 loops of biomass were suspended in 10 mL of autoclaved tap water. After vortexing for 10s, 100 µl of the inoculum were dispensed in HPSPA plates containing antibiotics. Plates were incubated in aerobic and microaerobic atmospheres at 23 and 37 °C, and checked for colony growth after 7 days.

4.2.7 Field studies

Field studies involved both planktonic and biofilm analysis using selective wHPA. For the planktonic studies samples were collected from lake, untreated fountain and tap waters. The lake from where the lake water was collected is located close to the Bom Jesus church in Braga, more precisely in the park surrounding the area at the 42° 33′ 29″ N, 8° 22′ 55″ W coordinates. It is an artificial lake with approximately 1000 m^2 with still waters and in a highly eutrophized state which gives it a green color. The fountain water was collected 20m in the north direction from the north border of the lake. The water comes from one of the many unpolluted streams that fill the park area. Tap water was

collected from the Applied Microbiology laboratory at the Center for Biological Engineering at the University of Minho. All samples were collected in October 2003 and immediately stored in the dark until further processing.

For the biofilm studies, pipes from real drinking water distribution systems from across Europe (already described in Section 2.2.8) were analyzed. The scraping technique mentioned in Section 3.2.4 was used to detach microorganisms from an area of approx. 1 cm^2 of the surfaces. For both studies, 100 μL of the samples were dispensed in wHPA plates with antibiotics (in triplicate), and incubated using the Campygen gas pack.

4.2.8 Confirmative procedures

Besides checking for typical colony morphology (round, translucent to yellowish, convex, 0.2-2 mm) (4), microscopic visualization with EDIC or EF microscopy (112) and hybridization with the specific peptide nucleic acid probe described in Chapter 2 was performed to confirm the existence of *H. pylori*.

4.2.9 Analysis of data

Nutrient shock results and all other time-series experiments were statistically analyzed employing a two-way or a one-way ANOVA. For all other comparisons, involving only one sample at a determined time of exposure, a t-test was used. Computations were performed using SPSS (SPSS Inc., USA), and results were considered statistically relevant if $P \leq 0.05$.

4.3 Results and discussion

4.3.1 Nutrient shock effects on *H. pylori* growth

HPSPA, CA and WCA, supplemented with 5% defribinated horse blood, were tested and compared with their diluted versions. For all media, results demonstrated that with half strength media higher recovery rates were achieved than with standard media (Fig. 4.2). A two-way ANOVA showed that these results were statistically relevant with $P<0.05$ for CA and $P<0.01$ for HPSPA and WCA. Besides giving higher recoveries on all of the

half strength media, half strength WCA was able to detect *H. pylori* for a longer period than the standard media. Quarter strength media failed to recover any bacteria, which was anticipated since *H. pylori* was not able to grow on these plates even when restreaked directly from the maintenance culture. Viable counts of *H. pylori* declined rapidly and recovery ended after 6 hours, which is consistent with earlier observations (219).

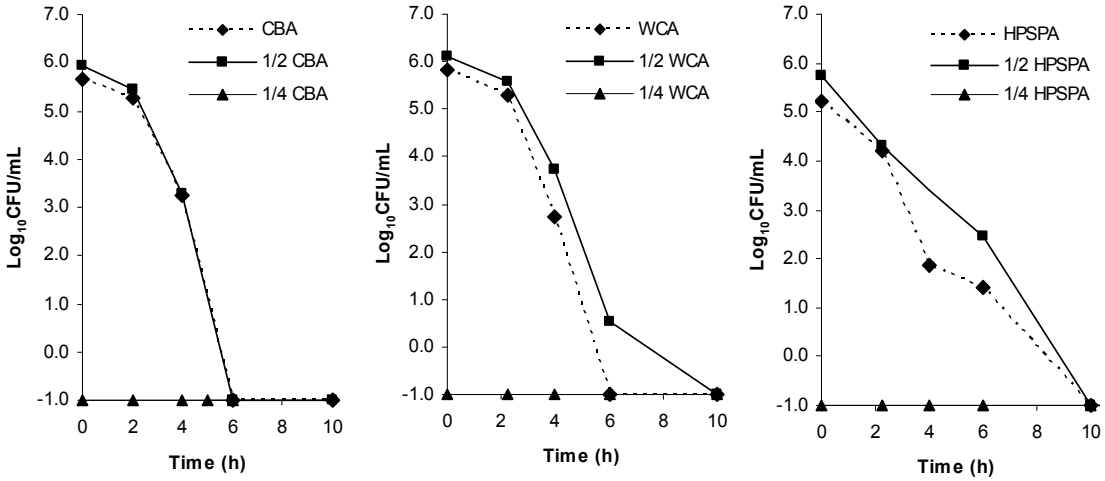


Fig. 4.2. Comparison between recoveries obtained from diluted and standard media. ANOVA analysis demonstrated a significant difference for all three figures between the full and half strength versions of the media ($P<0.05$).

In terms of colony size half strength WCA was found to be superior to the other half strength media as it produced consistently larger colonies. Half strength CBA was also found to be superior to half strength HPSPA. An example of how the colony size was distributed for the different media can be observed in Fig. 4.3. Similar profiles were obtained for different water exposure times. However, the size of colonies obtained for the full strength media did not appear to show much difference. Therefore, the differences obtained in the half strength media can be explained by the dilution factor, which probably decreases too much one essential component of CBA and HPSPA too much, making it insufficient to comply with the growth needs of the *H. pylori* colonies.

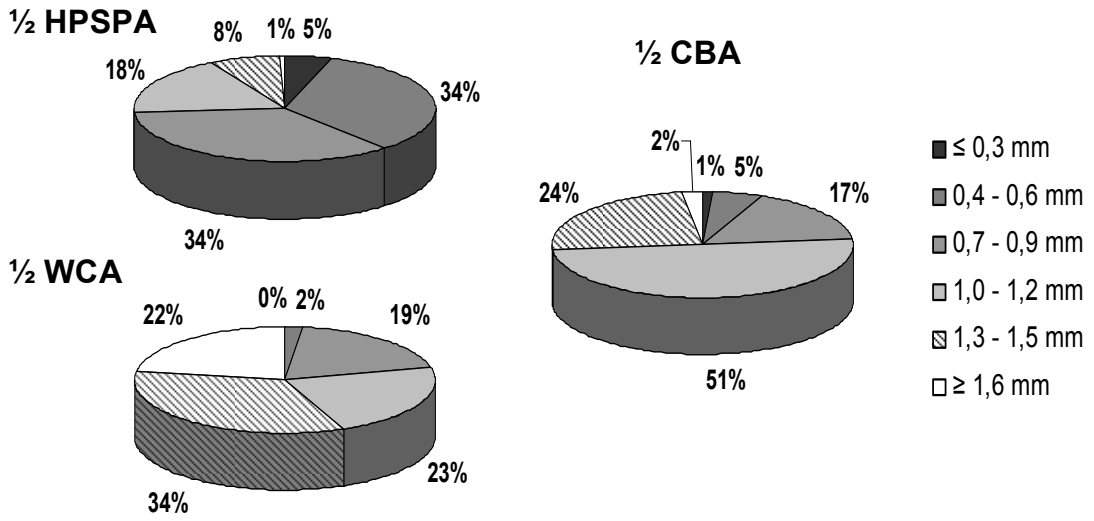


Fig. 4.3. Profile of colony sizes obtained after 7 days growth of *H. pylori* for the three different media. Sampling time t=0h.

The three media selected for this study have been used for *H. pylori* recovery from a range of varied conditions. CA has been, together with Brucella agar, extensively used in studies performed so far (4), whereas HPSPA was considered the most appropriate for the isolation of the pathogen from beef samples (233). WCA was employed in the recovery of the pathogen from ready-to-eat foods (195). The fact that half strength media gave higher recoveries than standard media indicate that nutrient shock is indeed a hampering factor in *H. pylori* recovery from low nutrient environments, and that possibly all other rich media employed in these type of systems for other types of microorganisms might indeed be underestimating the real number of viable cells. The quarter strength media failed to recover any bacteria. However, carbon content in these media is still very high when compared to R2A, the medium of choice for quantifying heterotrophic bacteria in low nutrient environments, such as water. This might denote that the lack of growth is due to the depletion of one or more essential growth components, other than carbon, during the growth of the colonies.

When compared to other bacteria, the number of regulatory proteins to respond to environmental stimuli in *H. pylori* is low, which might reflect the adaptation of the bacterium to the human gastric microenvironment (145). This could also imply a lack of ability from the bacteria to adjust and survive in different niches in the environment. This work suggests, however, that the bacteria can adapt physiologically to low nutrient environments, by showing that the direct recovery from water to a high nutrient media causes a nutrient shock. Moreover, a number of studies have already reported the mechanism of shape modification from spiral to coccoid as a protective mechanism against exposure to sub-optimal conditions (42, 170, 239). Altogether, this evidence points to the ability of *H. pylori* to adapt to niches other than the human gastric environment. A combination of proteomic and genomic studies is necessary to be carried out so that a further understanding of the mechanisms and proteins implicated in these changes can be gained.

4.3.2 Influence of the incubation atmosphere

The previous results indicate that nutrient shock is indeed a hampering factor in recovery of *H. pylori* from low nutrient environments. In order to investigate if the composition of the incubation atmosphere would also influence the recovery of *H. pylori* from water, several gas atmospheres were tested and compared. In initial experiments the oxygen concentration was varied (keeping a constant composition of carbon dioxide and hydrogen) to assess potential toxic effects. The CFU recovered was not significantly affected by the oxygen concentration between 1-13% but it was noted that colony size measured after 6 days of growth on normal CA medium decreased from 1 mm to 0.5 mm diameter in the more extreme concentrations.

The CampyGen system generates an atmosphere of 6% oxygen and 12% carbon dioxide and takes approx. 30 minutes to achieve. By contrast, the MACS workstation environment is user defined and instantaneous. It was decided from the oxygen experiments to keep its concentration at 5-6% as being optimal and also consistent with that of the CampyGen system. The efficiency of recovery was around 60% higher when using the MACS workstation compared to the CampyGen gas pack (Fig. 4.4), and statistical analysis

confirmed this difference was significant for all atmospheres tested ($P<0.01$). However, the composition of oxygen: carbon dioxide: hydrogen atmospheres within the range reported did not seem to make a statistically significant difference in the relative *H. pylori* recovery, when compared between themselves ($P>0.05$).

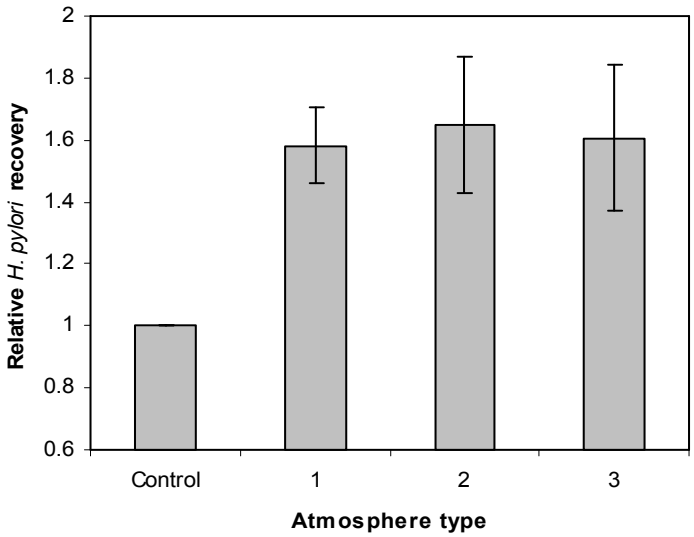


Fig. 4.4. Relative *H. pylori* recovery for different atmospheres when compared to recovery obtained by the use of Campygen sachets (control). Atmosphere 1 is 13% CO_2, 5% O_2, 3% H_2; atmosphere 2 is 5% CO_2, 5% O_2, 3% H_2 and atmosphere 3 is 15% CO_2, 6%O_2, 0% H_2. No statistically significative difference is found between the three described atmospheres.

Although gas packs are convenient in the maintenance of cultures, it became clear that for a more correct assessment of the number of viable *H. pylori* recovered from the environment a device is required that allows a more stable and user defined atmosphere during the incubation period. It appears that if oxygen concentrations are low enough, the concentrations of the other atmosphere constituents tested do not affect greatly the recovery of the pathogen. Oxygen toxicity is a detrimental factor when using CampyGen gas systems, as the generation of a suitable atmosphere takes approximately half an hour, during which time the frailest *H. pylori* turn into non-culturable cells. This means that an immediately achieved suitable incubation atmosphere is a more important factor to the

recovery of the pathogen than the composition of the atmosphere, at least between a certain range of oxygen:carbon dioxide:hydrogen concentrations.

4.3.3 Interaction between nutrient shock and atmosphere influence

To check whether the bacteria recovered by using the half strength plates was the same recovered by the use of the MACS workstation, a repetition of the previous experiment was done, using both CBA and half strength CBA agar plates (in quadruplicate). In the two experiments carried on, the numbers of *H. pylori* recovered with half strength CBA medium incubated in a MACS workstation was superior than the numbers obtained either using CBA medium in the variable atmosphere workstation or using half strength CBA medium in a CampyGen atmosphere, showing a synergy between both methods (Fig. 4.5).

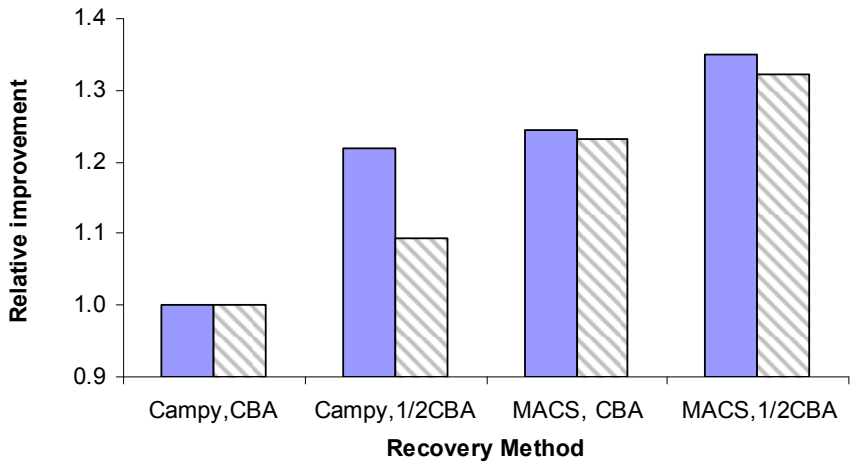


Fig. 4.5. Comparison between *H. pylori* recovered using a combination of different methods, with the MACS set to 5% CO_2, 5% O_2, 3% H_2 (full bars) and 7% CO_2, 7%O_2, 1% H_2 (dashed bars).

It was then observed that the recovery rate is improved when both methods are applied simultaneously. However, this increase might not be equal to the sum of the recoveries obtained separately, as some bacteria that can be recovered by half strength media can also be recovered by the use of a microaerophilic cabinet. Because the humidity in the MACS was set to 80% the relative improvement in this environment when compared to

CampyGen sachets was down to 20%. This highlights the importance of high humidity during incubation, a condition that will probably prevent the culture plates from drying more quickly and cause stress do the cells due to low water activity (51). It also implied that the only statistically relevant results found were between the CBA plates incubated using Campygen and the half strength CBA plates incubated in the MACS for both atmospheres ($P<0.05$).

4.3.4 Media development

Because the main objective in the first part of the work was to prove, or refute, the importance that a nutrient shock effect might have on *H. pylori* recovery from water, no attempts were made to optimize the constituents of the different media in terms of colony forming units and size. The work was however used as a basis to develop a suitable medium for the recovery of the pathogen from water or other nutrient-poor environmental samples. For this purpose, half strength versions of freshly made CA or WCA and HPSPA supplemented with 2.5% (v/v) horse blood were compared between them to check if any of the media provided an additional advantage. In terms of colony counts, no statistically significant differences were detected in any of the experiments ($P>0.05$) (Fig. 4.6). Adaptation to the maintenance culture medium appears not to have occurred. In terms of colony size, results were consistent with the ones reported in Fig. 4.3, where half strength WCA produced larger colonies than half strength CBA and HPSPA. Nevertheless, and because both the full strength media were similar in respect of colony size and individual constituents of HPSPA were readily available in the laboratory, HPSPA was chosen as the basis for a new low-nutrient medium.

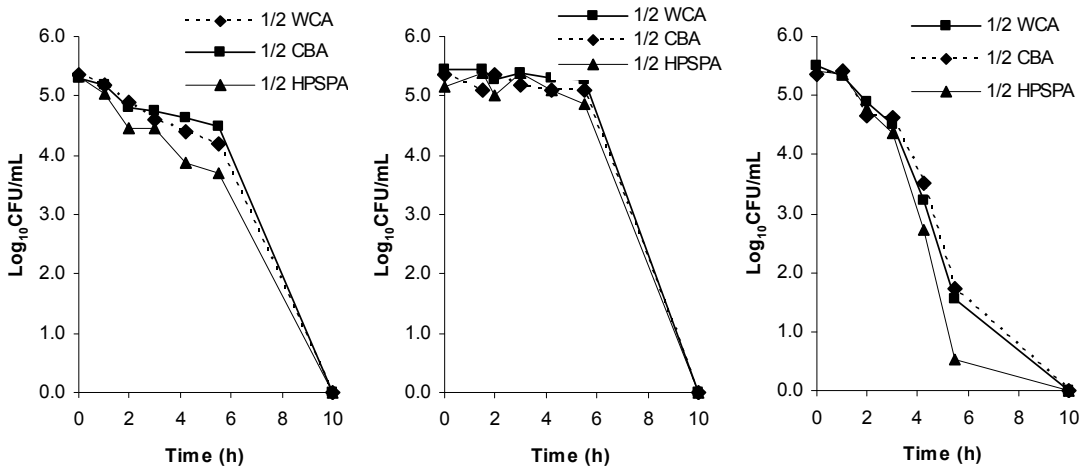


Fig. 4.6. Comparison between recoveries obtained for three different half strength media, for cells obtained from a WCA (left), HPSPA (center) and CBA (right) maintenance culture. ANOVA analysis demonstrated no significant difference for all three figures ($P>0.05$).

Sodium chloride was found to be an important constituent in HPSPA, not only to support growth but also in terms of colony size, and a strong positive correlation was observed between this parameter and sodium chloride concentration (Fig. 4.7). Standardized colony size was obtained by multiplying the colony size by nutrient content of the media used and dividing it by the nutrient content of the standard media (horse blood dry weight content not accounted for). Therefore, colony size was larger in the diluted medium with high sodium chloride concentration than in the standard medium. Of the remaining components, only yeast and meat extract at large concentrations appeared to support the growth of *H. pylori*, when all other components were reduced to a quarter of their normal concentration. However, the colony size obtained for the diluted medium in this case was smaller than the colony size obtained for HPSPA.

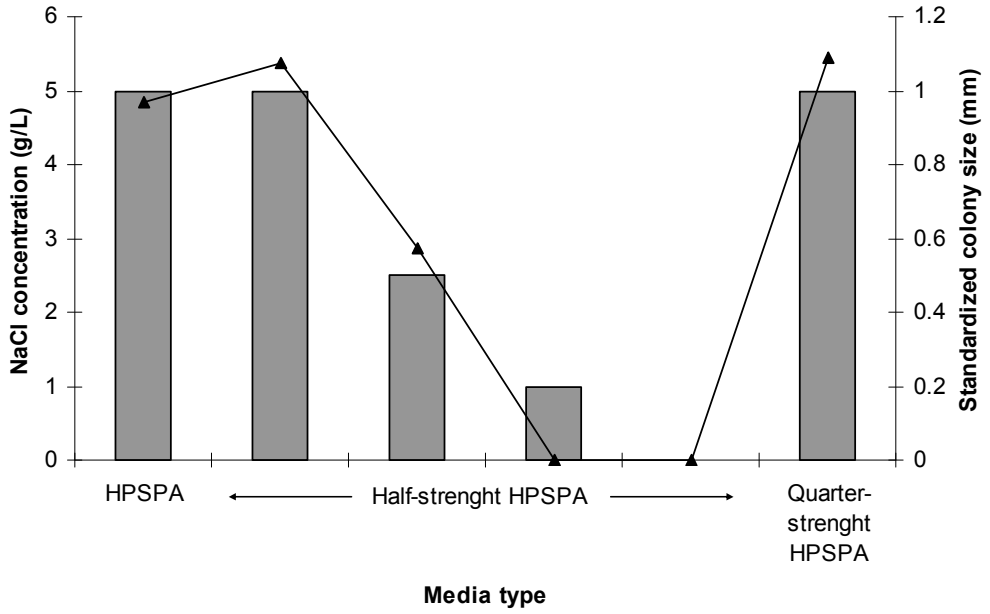


Fig. 4.7. Correlation between colony size after 7 days (line) and NaCl content in the medium (bars).

From the three new low nutrient media designed only wHPA yielded higher counts than HPSPA (Table 4.4), but even that result was not statistically significant (P=0.21). It is important to notice that the improvement of an average of 10% achieved when comparing HPSPA and the half-strength version was largely exceeded with wHPA. The other two media provided similar counts to HPSPA. As sodium concentrations were the same for all low-nutrient media, the poor behavior of wHPA(1) and wHPA(2) is probably due to a less-than optimum choice for the amounts of yeast and meat extract.

Table 4.4. Culturable cell counts obtained for HPSPA and three different low-nutrient media.

Medium	HPSPA	wHPA	wHPA(1)	wHPA(2)
Culturable counts (CFU/mL)	1.248×10^6	1.893×10^6	1.228×10^6	1.105×10^6

The efficiency of wHPA was due, in a large part, to maintaining the sodium chloride concentration used for HPSPA. In other reported studies, restriction of salt and salted food intake was suggested as a practical strategy to prevent gastric cancer in areas with high *H. pylori* prevalence (242), and suspicions of seawater as a transmission vehicle have recently arisen (35). Sodium chloride has also been implicated in pathogenesis as it potentiates the vacuolation toxin activity (139) and appears therefore to be a key element for *H. pylori* growth and metabolism.

Because the content of defribinated horse blood was also reduced, wHPA is a more transparent medium, which allows easier colony identification when compared to HPSPA (Fig. 4.8).

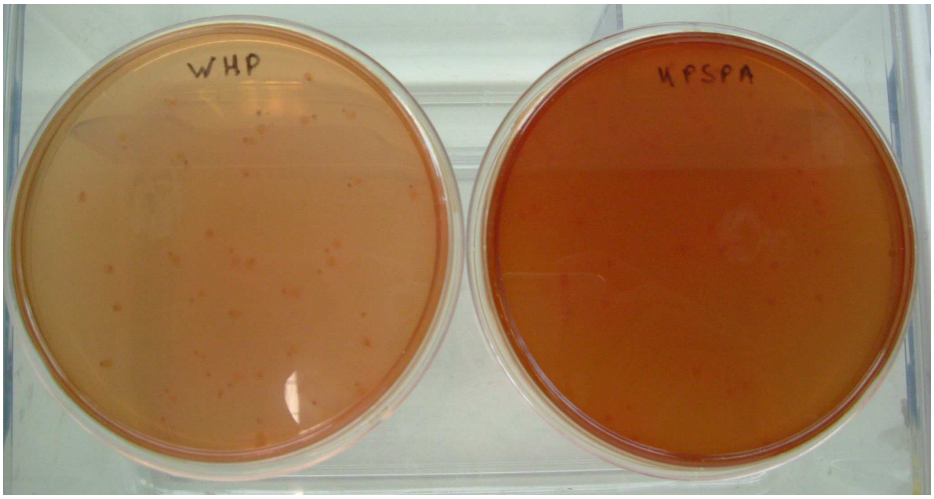


Fig. 4.8. Growth of *H. pylori* colonies in wHP (left) and HPSPA (right).

4.3.5 Media evaluation

The new medium wHPA provided better recoveries for 5 of the eight strains (Table 4.5). For 3 of the strains the recovery improved more than 30%, whereas all other strains stayed in the range of a -10% to 10% improvement. It is also important to notice that, of the strains where higher counts were obtained for HPSPA, 2 out of three presented P values higher than 0.9, meaning that statistically, the two media are probably similar in respect to *H. pylori* recovery from water.

Table 4.5. Percentage of improvement of HPSPA and wHPA media (n=3-12).

Strain	Provenience	Pathology	Percentage of improvement
1320	adult	Peptic ulcer	40%
968	child	Gastritis	-7%
1330	adult	Peptic ulcer	10%
957	child	Peptic ulcer	-3%
1198	child	Peptic ulcer	45%*
1152	child	Peptic ulcer	1%
1342	adult	Gastritis	-5%
NCTC 11637	type strain	-	34%*

* Statistically significant ($P<0.05$)

For NCTC 11637, the comparison of the culturable counts was also performed with time (data not shown). The ANOVA test demonstrated that, for this strain, the media were effectively different with time as well ($P<0.01$). This result was already expected because a statistically significant result had already been obtained between HPSPA and half-strength HPSPA (Section 4.3.1).

This work presents a reformulated medium with the specific purpose of recovering *H. pylori* from water. Perhaps the most striking result is the fact that not all strains appear to recover better in low nutrient medium, which may indirectly imply that while optimizing the medium for *H. pylori* NCTC 11637 one or more essential nutrients for some of the other strains has been decreased too much, preventing these strains from growing properly. An alternative interpretation is that adaptation to water does not occur for all of *H. pylori* strains. This may, however, not come as a surprise, because it has been demonstrated that this pathogen presents extensive strain and interstrain variation at both the genomic and the protein level (60). Differences in adaptation could actually suggest that *H. pylori* uses multiple routes to be transmitted from person to person, with non water-adapted species preferring other transmission routes, such as person-to-person. The existence of multiple transmission routes to infect human individuals has been suggested

by other authors as well (76, 154), and has also been observed for Campylobacter spp., a closely related species (230). Because it appears obvious that *H. pylori* strains that are able to adapt to environmental conditions will possess a better chance of being transmitted through the water, wHPA is likely to be very useful, even though it brings no obvious advantages apart from being more transparent, for the other strains tested in this study.

This work also shows that new microbial cultivation media need to be not only species-specific, but also adjusted to the environment the microorganism is recovered from. For the enumeration of heterotrophic microorganisms present in water or other low nutrient environments, it has already been shown that the low-nutrient medium R2A obtains much better results than other heterotrophic high-nutrient media (149, 199). Low nutrient medium efficiency is probably related to osmotic effects, which were considered to be the most important parameter for killing *H. pylori* in regionally different types of honey (183). Also, Enroth and co-workers have shown that coccoid forms of *H. pylori* have lower density than the spiral counterparts (61). As it has been shown that transformation from spiral to coccoid is a common process when the microorganism is exposed to water, it is possible that this is in fact a response mechanism of the bacterium to withstand the osmotic effects caused by exposure to water.

4.3.6 Alternative strategies to improve the medium

Filtration of water samples to concentrate microorganisms and grow them in agar has been a widely applied strategy in microbiology. Results obtained in this study showed that the relative recovery of the filtration technique compared to the standard technique (after accounting for the different volumes dispensed) was 0.064, 0.059 and 0.129 for the 0.1, 0.2 and 0.45 μm filters, respectively. It would therefore be necessary to filter ca. 16 times (for 0.1 and 0.2 μm filters) and ca. 8 times (for 0.45 μm filters) more water than the dispensed quantity in the plate to achieve the same CFU counts. It has been suggested that, because *H. pylori* has a relatively small size and is motile, it could swim through the filter membranes (A. Lastovica, personal communication), which could explain the low amount of *H. pylori* recovered. However, if this was the case, then one would expect that recovery would increase with decreasing pore sizes, which is actually the reverse of what

happens. The real reason for the low recovery rate obtained by the filtration method is then probably the lack of access of *H. pylori* to the essential nutrients in the agar. The larger recovery in the 0.45 μm filter would then be explained by an easier diffusion of those nutrients through larger pores.

Stressed bacteria tend to take longer to grow than physiologically active bacteria, however, extending the time of incubation did not cause an improvement in the recovery of *H. pylori*, as all colonies appeared within 7 days. Longer incubation times have been observed for a large array of fastidious bacteria, including *Bartonella* sp., which usually takes 14-50 days to form visible colonies, and *Mycobacterium sp.* that can take up to 8 weeks to grow. Replacing NaCl by KCl in HPSPA also yielded ca. 5 times less growth of *H. pylori*. Other techniques to improve HPSPA (addition of porcine mucin, ferrous sulfate, sodium pyruvate) have also been tried by other authors with no success (52).

4.3.7 Evaluation of the antibiotics impact

The results obtained when HPSPA with antibiotics was tested against microorganisms that have been isolated from drinking water biofilms are presented in Table 4.6. As it can be observed, when incubated in the necessary conditions for *H. pylori* to grow, selective HPSPA still supported growth of three bacteria, namely *Methylobacterium mesophilicum*, *Burkholderia cepacia* and *Flavimonas oryzihabitans*. None of these bacteria has been included as a test-species in the article that originally described and evaluated the selective medium (52). Growth of these species in the selective medium shows that there is still room for improvement in the development of a selective supplement for *H. pylori* in water.

Aditional microorganisms were also able to grow if selective HPSPA was incubated at 23°C under aerobic conditions, implying that both elevated temperature and the incubation atmosphere cause a greater selective pressure on certain bacteria such as *Pseudomonas fluorescens, Comamonas acidovorans, Moraxella lacunata* and *Brevundimonas diminuta*. All bacteria except *Sphingomonas paucimobilis* were able to grow in non-selective CBA at the same incubation conditions.

Table 4.6. Evaluation of growth from heterotrophic bacteria in the *H. pylori* selective medium HPSPA with antibiotics and in the non-selective CBA at different temperatures and atmospheres.

Microorganism	Aerobic (23 °C)		Microaerobic (37 °C)	
	CBA	HPSPA	CBA	HPSPA
E. coli	+	-	n. p.	-
Pseudomonas fluorescens	+	+	-	-
Stenotrophomonas maltophilia	+	-	+	-
Sphingomonas paucimobilis	-	-	-	-
Comamonas acidovorans	+	+/-	n. p.	-
Moraxella lacunata	+	+	-	-
CDC gr.IV C-2[a]	+	-	+	-
Brevundimonas diminuta	+	+	+	-
Methylobacterium mesophilicum	+	+	+	+
Burkholderia cepacia	+	-	+	+/-
Brevundimonas vesicularis	+	-	+	-
Aeromonas salmonicida	+	-	+	-
Acinetobacter lwoffi	+	-	+	-
Flavimonas oryzihabitans	+	-	+	+/-
H. pylori	-	-	+	+

+Good growth; +/-Poor growth; -No growth; n. p. Not performed

[a]Identified by the Centers of Disease Control and Prevention (CDC) as a Class Two microorganism, and hence prejudicial to human health.

The effect of the addition of antibiotics already described in the literature (52) to wHPA was also studied. In this new experiment, the percentage of improvement for *H. pylori*

NCTC 11637 strain was 31.5% between wHPA and HPSPA with no antibiotics, and 16.3% between wHPA and HPSPA with antibiotics. The antibiotics at the concentrations described in Degnan *et al*. (52) appear therefore to cause a small deleterious effect on the growth of *H. pylori*. Even though wHPA with antibiotics is still superior to HPSPA with antibiotics, a reassessment of the concentrations of each of the compounds in the selective supplement might be necessary to further enhance the medium performance.

4.3.8 Field studies

In the planktonic studies, no *H. pylori* was detected in any of the samples. One colony from a contaminating microorganism was observed in the tap water sample, and there was also unacceptable overgrowth from contaminating microorganisms in the sample from the lake, and occasional contamination on the fountain sample. This evidence coincides with the lab data that indicated that some of the aquatic microflora was still able to grow on the selective medium. Further development of a selective supplement needs therefore to be performed, especially if the medium is intended to be applied to heavily contaminated and untreated water samples.

For the biofilm studies, no *H. pylori* was also found. Contrary to the planktonic sample analysis, contaminants were not observed in any of the samples. These results can be easily explained by the fact that biofilms were formed when exposed to treated water containing chlorine, and also due to the large processing time of the samples (3 hours for Portuguese samples and up to 3 days to international samples). Even though this large processing time was not expected to influence in the PNA analysis, culturability is much more dependent on this parameter.

Studies to recover viable *H. pylori* from drinking water and drinking water associated biofilms have been unsuccessful so far, and the results obtained in the field studies had therefore been antecipated. Even if *H. pylori* is not consistently present in drinking water systems, the fact that the colonization does not cause disease immediately after contamination occurs implies that detection will be more difficult. A next logical step for this work could therefore be to include screening of the pathogen during indicator bacteria

failure in drinking water systems, where increased chances of detection exist, using this new medium and the incubation technique.

4.4 Conclusions

Although substantial evidence has been found linking *H. pylori* infection among human population with water consumption, plate spreading techniques have so far failed to recover this pathogen from drinking water systems. For contaminated drinking water to be accepted as a route of transmission it is important to successfully cultivate the pathogen from these type of systems. The new low-nutrient medium wHPA yields higher counts than previously employed media, but the time that the bacteria can be detected remains practically unchanged (6 to 20 hours at 24 °C), meaning that the pathogen culturability is still significantly lower than for other pathogenic bacteria known to be transmitted through the water. Because it was concluded that cells can be stressed in different ways, and that a good recovery process has to employ a combination of both a suitable medium and proper incubation conditions, it is recommended that the agar plates are incubated in an atmosphere of 5% CO_2, 5% O_2 and 3% H_2, although other atmospheres with a low atmosphere concentration are acceptable.

For recovery to occur properly it is still needed to improve selectivity of the medium, processing times of the field samples and, in the case of biofilm samples, develop a more efficient technique for the detachment of the bacteria from the surfaces.

5 General conclusions

The main goal of this thesis was to clarify the role of water and water-associated biofilms as sources of infection of *H. pylori,* and consequently provide information that allows the design of strategies to prevent the infection of human individuals in the future. This route of transmission has been implicated by several epidemiological studies and is furthermore supported by the systematic findings of *H. pylori*-specific DNA/RNA sequences in real water and drinking water biofilms using molecular biology techniques, such as FISH or PCR. However, the survival of the bacterium in water is quite low and its recovery from real drinking water systems using culture techniques remains elusive. The three hypotheses that were at the time more likely to explain these apparent discrepancies were the framework of this work. To know: the time of survival will increase if *H. pylori* is interacting with microorganisms of the same or other species in the complex structures formed on pipe or other types of surfaces in contact with water; the techniques used to recover the bacteria do not detect VBNC bacteria still capable of causing infection; or the bacteria detected by molecular techniques are "dead" and/or molecular techniques are detecting yet uncultured microorganisms with similar nucleic acid sequences. Pros and cons of each of the theories are now analysed in detail, under the new light shed by the results obtained in this thesis.

Theory 1: Interaction with microorganisms of the same or other species in biofilms formed in the water-exposed surfaces extends *H. pylori* survival

PNA FISH information about *H. pylori* presence in heterotrophic biofilms showed that the pathogen is able to attach directly to the surface or migrate close to the basal layer of the biofilm. The large autofluorescence observed in stacks or fronds prevented an unequivocal detection of the pathogen in these structures. Dual-labelling of the pathogen with the PNA probe and viability dyes was also not performed due to the same problem. Important information about the physiological state of *H. pylori* in heterotrophic environments was therefore not obtained, but the data collected allow speculation that certain microorganisms can provide low redox zones where the pathogen will have increased chances of survival. Free-living amoebae, which are typical predators found in

biofilm ecosystems, were already found to provide conditions favouring the survival of *H. pylori* (263). On the other hand, the apparent inability of the bacteria to maintain membrane integrity and culturability status in monospecies biofilms, despite the formation of aggregates and maintenance of spiral morphology, indicates that no obvious advantage for survival occurs when pure-culture biofilms are formed on abiotic surfaces. It is important to bear in mind, however, that this interpretation of the results is largely dependent on the suitability of membrane integrity and culture techniques as indicators of cell viability.

Assuming that *H. pylori* can indeed survive for longer when in contact with protozoa or other microorganisms from heterotrophic biofilms, it is important to understand under what conditions the bacterium is able to incorporate in these structures. It has been shown in this work that *H. pylori* attaches better to surfaces under static conditions, pointing towards well water as one of the most plausible areas from where culture of the bacteria is possible. Interestingly, research groups concerned with *H. pylori* transmission have been giving particular attention to well water in the last few years, and many of them independently concluded that drinking from this type of water supply is a risk factor for the acquisition of the pathogen (8, 95, 107, 123).

If this theory is correct, the lack of a suitable selective plating media would be the main reason for the pathogen not to be detected by culturable methods in well water. Flanigan and Rodgers claimed to have cultured *H. pylori* from well water in 2003, but because of overgrowth by faster growing background bacteria, the growth of the pathogen could only be detected by coupling the culture method with PCR techniques (64). As large scale surveys are being currently carried out with proper selective media, the accuracy of this detection should be verified soon.

Theory 2: Lack of adequate recovery techniques

This work reported a nutrient shock effect when recovering the water-stressed bacterium on high nutrient medium, showing some level of *H. pylori* adaptation to this environment. However, the time the bacterium remained culturable after exposure to the bulk fluid was nearly constant, even after a new low-nutrient medium was developed. Other parameters

that were studied to try to increase this same parameter (such as different incubation atmospheres, humidity or the use of filter pads to extend incubation time) did not appear to influence it greatly.

While *H. pylori* culturability usually ends after a short time, Shahamat *et al.* (219) determined that the total bacterial cell counts did not decrease over much longer periods (2 years at 4 °C), and Adams *et al.* (1) revealed that *H. pylori* also maintained membrane integrity despite loss of culturability. Furthermore, She *et al.* (221) demonstrated that coccoid *H. pylori* induced by water is capable of colonizing the gastric mucosa and cause gastrititis in mice. In the same work, the authors clearly state that coccoid morphology is associated to non-culturability, but fail to use other viability methods (such as membrane integrity or the CTC method) to determine if any of them could closely reveal the true *H. pylori* infectious nature. Nevertheless, the study suggests that there are strong possibilities that currently employed culture methods are failing to detect viable bacteria. Under this theory, it is therefore imperative to better understand what transformations occur when *H. pylori* turns into the non-culturable state, so that a rational basis is provided for the development of new recovery techniques.

Theory 3: *H. pylori* is in a dead state and/or molecular techniques are not species specific

Small-scale field studies performed in this work have shown that *H. pylori* will not be easily identified in water supplies and associated biofilms, even after employing a selective culture medium that acceptably reduces the growth of other bacteria. Lack of detection using standard culture techniques is perhaps the most compelling evidence to support the view that *H. pylori* is non-viable in water and therefore not transmitted through this route. Even though other viability methods based on fluorescence techniques have been developed and accepted by most of the scientific community, it has been shown here that coupling these methods with PNA FISH probes to detect the pathogen in real heterotrophic microenvironments is still technically unfeasible. Use of the Syto9/PI technique showed however that the membrane of attached bacteria starts to become compromised after 48h at 23 °C, which is still not a big increase in terms of survival time.

Proof that culture methods are not appropriate was provided when She *et al.* reported in their work that coccoid (and unculturable) *H. pylori* could still cause infection in mice (221). Supporters of this theory can however still take comfort from the fact that this experiment has never been repeated since. On the other hand, techniques based on molecular biology rely heavily on the uniqueness of the DNA sequence they are tracking to identify *H. pylori*. Still, it is estimated that only 0.01 to 0.1% of all the microbial cells from aquatic environments form colonies on standard agar plates (63). Many of these are bacteria that have never been isolated which indicates that plenty of DNA/RNA sequences are still to be determined.

At this point of research, is it acceptable to state that water is an environmental reservoir for *H. pylori*?

The possibility of *H. pylori* being transmitted through the water is also determined by the existence of alternative vehicles or modes of transmission. Apart from the gastro-oral transmission, researchers investigating all other routes face the same problems concerning the culturability of the pathogen. Timing to perform the sampling is one of the issues, as infection does not immediately cause disease and only one exposure appears enough to cause infection. Gastro-oral transmission becomes nevertheless the only strong direct route of transmission, but it is unlikely that it accounts for such high levels of prevalence worldwide.

Even if water used to be a major environmental reservoir for *H. pylori* subsistence, the drop in the levels of prevalence for the last 20 years in the more developed countries would indicate that the disinfection procedures carried out by drinking water companies prevented the bacteria from being transmitted through this route, unless a disinfection failure occurs in the system. Well water and other untreated water supplies appear to be, however, strong candidates as extragastric reservoirs harbouring *H. pylori*.

5.1 Future work

Based on the conclusions derived from this thesis, the future work to be carried out can continue to be performed in two major areas. The first will evaluate if *H. pylori* fits in the

definition of "viable but non culturable" bacterium when exposed to water, while the second will test whether any increase in culturability is achieved by co-aggregation with endogenous aquatic bacteria. A more detailed explanation of how to perform these tests follows:

Assessment of the viability of water-exposed H. pylori using animal models

This task assesses whether water-stressed cells not detected by current culture methodologies are able to cause infection in animal models. Therefore, *H. pylori* in the exponential phase is stressed by water and samples will be taken at different times of exposure. These samples will then be either: a) Plated on a standard *H. pylori* medium, such as Columbia Blood Agar; b) Plated in the recently developed low-nutrient medium that allows the recovery of higher numbers of certain *H. pylori* strains from water; c) Stained with DAPI for total cell counts; d) Stained with LIVE/DEAD® and/or hybridized with the specific 16S rRNA PNA probe for the assessment of membrane integrity or physiological status (ribosomal content) and e) Inoculated in mouse models to observe the colonization and infectivity ability. This task indicates if unculturable bacteria still retain their ability to cause infection and, if so, whether this ability is better correlated with one of the other detection methods (DAPI, LIVE/DEAD® or PNA FISH).

Comparative proteomics

The molecular mechanisms involved in the adaptation of *H. pylori* to water (as observed by the morphological change and the nutrient shock) are able to be studied using proteomics. For that, a quantitative comparison between the exponentially-growing, lag phase and water-stressed *H. pylori* (exposed at different times) proteome could be performed. Novel proteomic technologies, such as iTRAQ® (90), are now starting to be used as more efficient ways to quantify, in absolute terms, the proteins of interest. Simultaneous comparison of individual protein expression levels from cells of different physiological status (e.g. viable vs. non-viable) can also be performed by this technique. The physiological function of proteins with different levels of expression can thereafter be

identified using computer analysis. Data collected from these studies will hopefully serve to further enhance the new culture medium that has been developed in this work.

Enhancement of the new medium for water-stressed H. pylori

Although the recovery rates are higher for some strains of *H. pylori*, the culturability time of the water-exposed bacterium remains the same. The design of a new medium will exploit the information collected on the comparative proteomics work in an attempt to identify the missing component(s) that hinders *H. pylori* growth. Suitability of the medium to water can also be tested by adapting the selectivity agents described in Degnan *et al.* (52). Obviously, this task is only to be executed if in the animal model studies it is shown that the culture media available does not detect infectious *H. pylori*.

Adhesion and co-aggregation of H. pylori

Dynamic adhesion of *H. pylori* to different materials can be studied employing the two-stage chemostat system already developed, and compared to the results obtained by static adhesion. These studies are therefore to be performed in pure culture to avoid the interference of endogenous water bacteria in the results. After inoculation in the system, the bacteria can be tracked both by DAPI and culture methods. Architecture of the biofilm and the shape of the cells is easily observed under scanning electron and a confocal laser scanning microscopy (CSLM). If considered relevant, the same system and methods might also be applied to study the effect of other parameters in *H. pylori* adhesion.

Methodologies already described in Buswell *et al.* (34) can be employed in the co-aggregation tests. The other species used can be isolated from drinking water systems using R2A agar and identified by sequencing the 16S rRNA. In addition to the tests already described in the literature, the water suspension containing the two bacteria can be filtered and stained with DAPI and PNA FISH (dual-staining). This will provide special and architectural information about the preferred binding places for the different bacteria and *H. pylori*.

Field studies

This task uses the methodologies and information gathered in the other tasks to assess the prevalence of *H. pylori* in drinking water distribution systems and wells. The detection can be either performed *in situ* (using PNA FISH technology), or using the new culture plating techniques after the scraping of the biofilm. Critical points for the prevalence of the bacteria, as indicated by the adhesion assays, deserve a particular attention. If the co-aggregation study shows a particularly increased survival of *H. pylori* when co-aggregated with a certain bacterium, it is important that prevalence of such a bacterium is monitored and considered to be a risk factor. The presence or absence of *H. pylori* can be correlated with relevant parameters of the drinking water system, such as flow velocity (shear stress), pipe material, temperature, microbiological profile and chlorine concentration among others.

6 Scientific output

Parts of the results presented in this thesis have been published elsewhere:

Book Chapters:

Azevedo, N. F., M. J. Vieira, and C. W. Keevil. 2003. Development of peptide nucleic acid probes to detect *Helicobacter pylori* in diverse species potable water biofilms, p. 105-112. *In* A. McBain, D. Allison, M. Brading, A. Rickard, J. Verran, and J. Walker (ed.), Biofilm Communities: Order From Chaos? BioLine, Cardiff.
Azevedo, N. F., M. J. Vieira, and C. W. Keevil. 2005. Development of an optimized technique for the recovery of *Helicobacter pylori* from water and drinking water biofilms. (accepted).

Papers in peer reviewed journals:

Azevedo, N. F., A. P. Pacheco, M. J. Vieira, and C. W. Keevil. 2004. Nutrient shock and incubation atmosphere influence recovery of culturable *Helicobacter pylori* from water. Applied and Environmental Microbiology **70:**490-493.
Azevedo, N. F., M. J. Vieira, and C. W. Keevil. 2003. Establishment of a continuous model system to study *Helicobacter pylori* survival in potable water biofilms. Water Science and Technology **47:**155-160.

Proceedings and abstracts:

Azevedo, N. F., Pacheco A. P., Pinto A. R., C. W. Keevil, and M. J. Vieira. 2004. Study of surface characteristics of water-exposed *Helicobacter pylori* and plumbing materials. Presented at the International Conference Biofilms 2004: Structure and Activity of Biofilms, Las Vegas – U.S.A. Proceedings of Biofilms 2004, 115:118.
Azevedo, N. F., Pacheco A. P., Chaves L. C., C. W. Keevil, and M. J. Vieira. 2004. Detection and recovery of *Helicobacter pylori* from water and water-associated biofilms. Presented at the 10th International Symposium on Microbial Ecology ISME-10, Cancun – México. ISME-10 Book of Abstracts, 14.
Azevedo, N. F., M. J. Vieira, and C. W. Keevil. 2003. In vitro study of *Helicobacter pylori* association to pre-established drinking water biofilms. Presented at the Biofilms 2003 ASM Conferences, Victoria – Canada. Book of Abstracts, 165.
Azevedo, N. F., A. P. Pacheco, M. J. Vieira, and C. W. Keevil. 2003. Can the recovery of *Helicobacter pylori* from water be improved? Presented at the 12th International Workshop on Campylobacter, Helicobacter and Related Organisms, Aarhus – Denmark. International Journal of Medical Microbiology **293(S35):**85.
Azevedo, N. F., M. J. Vieira, and C. W. Keevil. 2002. Establishment of a continuous model system to study *Helicobacter pylori* survival in potable water biofilms. Presented at the International Specialised Conference on Biofilm Monitoring, Porto – Portugal. Proceedings of Biofilms 2002, 112:114.

7 References

1. **Adams, B. L., T. C. Bates, and J. D. Oliver.** 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. Applied and Environmental Microbiology **69:**7462-7466.
2. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.
3. **Alm, R. A., and T. J. Trust.** 1999. Analysis of the genetic diversity of *Helicobacter pylori*: the tale of two genomes. Journal of Mol Medicine **77:**834-46.
4. **Anderson, L. P., and T. Wadstrom.** 2001. Basic bacteriology and culture, p. 27-38. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
5. **Artz, R. R. E., and K. Killham.** 2002. Survival of *Escherichia coli* O157 : H7 in private drinking water wells: influences of protozoan grazing and elevated copper concentrations. FEMS Microbiology Letters **216:**117-122.
6. **Assanta, M. A., D. Roy, M. J. Lemay, and D. Montpetit.** 2002. Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. Journal of Food Protection **65:**1240-1247.
7. **Assanta, N. A., D. Roy, M. J. Lemay, and D. Montpetit.** 2002. Evidence for *Escherichia coli* O157 : H7 attachment to water distribution pipe materials by scanning electron microscopy. Journal of Food Protection **65:**1970-1975.
8. **Baker, K. H., and J. P. Hegarty.** 2001. Presence of *Helicobacter pylori* in drinking water is associated with clinical infection. Scandinavian Journal Infectious Diseases **33:**744-746.
9. **Baker, K. H., J. P. Hegarty, B. Redmond, N. A. Reed, and D. S. Herson.** 2002. Effect of oxidizing disinfectants (chlorine, monochloramine, and ozone) on *Helicobacter pylori*. Applied and Environmental Microbiology **68:**981-984.
10. **Bakka, A. S., and B. A. Salih.** 2002. Prevalence of *Helicobacter pylori* infection in asymptomatic subjects in Libya. Diagnostic Microbiology and Infectious Disease **43:**265-268.
11. **Baron, S., R. C. Peake, D. A. James, M. Susman, C. A. Kennedy, M. J. D. Singleton, and S. Schuenke.** 1996. Medical Microbiology. The University of Texas Medical Branch at Galveston, Galveston.
12. **Baschong, W., R. Suetterlin, and R. H. Laeng.** 2001. Control of autofluorescence of archival formaldehyde-fixed, paraffin-embedded tissue in confocal laser scanning microscopy (CLSM). Journal of Histochemistry & Cytochemistry **49:**1565-1571.
13. **Beatty, M. E., T. Jack, S. Sivapalasingam, S. S. Yao, I. Paul, B. Bibb, K. D. Greene, K. Kubota, E. D. Mintz, and J. T. Brooks.** 2004. An outbreak of *Vibrio cholerae* O1 infections on Ebeye Island, Republic of the Marshall Islands,

associated with use of an adequately chlorinated water source. Clinical Infectious Diseases **38:**1-9.

14. **Bjorkholm, B., M. Sjolund, P. G. Falk, O. G. Berg, L. Engstrand, and D. I. Andersson.** 2001. Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. Proceedings of the National Academy of Sciences of the United States of America **98:**14607-14612.
15. **Blaser, M. J., and J. C. Atherton.** 2004. *Helicobacter pylori* persistence: biology and disease. Journal of Clinical Investigation **113:**321-333.
16. **Blecker, U., S. Lanciers, E. Keppens, and Y. Vandenplas.** 1994. Evolution of *Helicobacter pylori* positivity in infants born from positive mothers. Journal Pediatr Gastroenterol Nutr **19:**87-90.
17. **Bode, G., D. Rothenbacher, H. Brenner, and G. Adler.** 1998. Pets are not a risk factor for *Helicobacter pylori* infection in young children: results of a population-based study in Southern Germany. Pediatric Infectious Disease Journal **17:**909-912.
18. **Boe-Hansen, R., H. J. Albrechtsen, E. Arvin, and C. Jorgensen.** 2002. Bulk water phase and biofilm growth in drinking water at low nutrient conditions. Water Research **36:**4477-4486.
19. **Boomkens, S. Y., J. G. Kusters, G. Hoffmann, R. G. J. Pot, B. Spee, L. C. Penning, H. F. Egberink, T. S. G. A. M. van den Ingh, and J. Rothuizen.** 2004. Detection of *Helicobacter pylori* in bile of cats. FEMS Immunology and Medical Microbiology **42:**307-311.
20. **Bos, R., H. C. van der Mei, and H. J. Busscher.** 1999. Physico-chemistry of initial microbial adhesive interactions - its mechanisms and methods for study. FEMS Microbiology Reviews **23:**179-230.
21. **Boulos, L., M. Prevost, B. Barbeau, J. Coallier, and R. Desjardins.** 1999. LIVE/DEAD (R) BacLight (TM): application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. Journal of Microbiological Methods **37:**77-86.
22. **Bovill, R. A., and B. M. Mackey.** 1997. Resuscitation of 'non-culturable' cells from aged cultures of *Campylobacter jejuni*. Microbiology-Uk **143:**1575-1581.
23. **Branda, S. S., A. Vik, L. Friedman, and R. Kolter.** 2005. Biofilms: the matrix revisited. Trends in Microbiology **13:**20-26.
24. **Breeuwer, P., and T. Abee.** 2000. Assessment of viability of microorganisms employing fluorescence techniques. International Journal of Food Microbiology **55:**193-200.
25. **Briandet, R., T. Meylheuc, C. Maher, and M. N. Bellon-Fontaine.** 1999. *Listeria monocytogenes* Scott A: Cell surface charge, hydrophobicity, and electron donor and acceptor characteristics under different environmental growth conditions. Applied and Environmental Microbiology **65:**5328-5333.
26. **Brown, D. A., T. J. Beveridge, C. W. Keevil, and B. L. Sheriff.** 1998. Evaluation of microscopic techniques to observe iron precipitation in a natural microbial biofilm. FEMS Microbiology Ecology **26:**297-310.
27. **Broxmeyer, L.** 2004. Heart disease: the greatest 'risk' factor of them all. Med Hypotheses **62:**773-9.

28. **Brummer, I. H. M., A. Felske, and I. Wagner-Dobler.** 2003. Diversity and seasonal variability of beta-proteobacteria in biofilms of polluted rivers: Analysis by temperature gradient gel electrophoresis and cloning. Applied and Environmental Microbiology **69:**4463-4473.
29. **Bumann, D., H. Habibi, B. Kan, M. Schmid, C. Goosmann, V. Brinkmann, T. F. Meyer, and P. R. Jungblut.** 2004. Lack of stage-specific proteins in coccoid *Helicobacter pylori* cells. Infection and Immunity **72:**6738-6742.
30. **Bunn, J. E. G., W. G. MacKay, J. E. Thomas, D. C. Reid, and L. T. Weaver.** 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. Letters in Applied Microbiology **34:**450-454.
31. **Busscher, H. J., A. H. Weerkamp, H. C. Vandermei, A. W. J. Vanpelt, H. P. Dejong, and J. Arends.** 1984. Measurement of the Surface Free-Energy of Bacterial-Cell Surfaces and Its Relevance for Adhesion. Applied and Environmental Microbiology **48:**980-983.
32. **Buswell, C. M., Y. M. Herlihy, C. W. Keevil, P. D. Marsh, and S. A. Leach.** 1999. Carbon load in aquatic ecosystems affects the diversity and biomass of water biofilm consortia and the persistence of the pathogen *Campylobacter jejuni* within them. Journal of Applied Microbiology **85:**161s-167s.
33. **Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. McGuiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach.** 1998. Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Applied and Environmental Microbiology **64:**733-741.
34. **Buswell, C. M., Y. M. Herlihy, P. D. Marsh, C. W. Keevil, and S. A. Leach.** 1997. Coaggregation amongst aquatic biofilm bacteria. Journal of Applied Microbiology **83:**477-484.
35. **Cellini, L., A. Del Vecchio, M. Di Candia, E. Di Campli, M. Favaro, and G. Donelli.** 2004. Detection of free and plankton-associated *Helicobacter pylori* in seawater. Journal of Applied Microbiology **97:**285-292.
36. **Chao, W. L., R. J. Ding, and R. S. Chen.** 1988. Survival of *Yersinia enterocolitica* in the Environment. Canadian Journal of Microbiology **34:**753-756.
37. **Chaves, L.** 2005. Estudo da cinetica da formacao de biofilmes em superficies em contacto com a agua potavel. PhD thesis. University of Minho, Braga.
38. **Cittelly, D. M., M. P. Dore, M. M. Bravo, H. Monsalve, R. Botero, O. Ricaurte, Y. Yamaoka, H. M. T. El-Zimaity, M. S. Osato, J. G. Fox, G. Realdi, and D. Y. Graham.** 2002. *Helicobacter pylori* in animals is of human origin: studies in monkey's, sheep, and cats. Gut **51:**A31-A31.
39. **Citterio, B., A. Casaroli, L. Pierfelici, M. Battistelli, E. Falcieri, and W. Baffone.** 2004. Morphological changes and outer membrane protein patterns in *Helicobacter pylori* during conversion from bacillary to coccoid form. New Microbiology **27:**353-60.
40. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner

that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Research **31:**442-443.

41. **Cole, S. P., J. Harwood, R. Lee, R. She, and D. G. Guiney.** 2004. Characterization of monospecies biofilm formation by *Helicobacter pylori*. Journal of Bacteriology **186:**3124-3132.
42. **Cole, S. P., V. F. Kharitonov, and D. G. Guiney.** 1999. Effect of nitric oxide on *Helicobacter pylori* morphology. Journal of Infectious Diseases **180:**1713-1717.
43. **Combe, E. C., B. A. Owen, and J. S. Hodges.** 2004. A protocol for determining the surface free energy of dental materials. Dental Materials **20:**262-268.
44. **Connon, S. A., and S. J. Giovannoni.** 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Applied and Environmental Microbiology **68:**3878-3885.
45. **Constanza, C. M., L. P. Eduardo, T. Javier, V. M. Eduardo, Q. Manuel, and C. Pelayo.** 2004. Determinants of *Helicobacter pylori* seroprevalence in Mexican adolescents. *Helicobacter* **9:**106-14.
46. **Cools, I., M. Uyttendaele, C. Caro, E. D'Haese, H. J. Nelis, and J. Debevere.** 2003. Survival of *Campylobacter jejuni* strains of different origin in drinking water. Journal of Applied Microbiology **94:**886-892.
47. **Costerton, J. W., G. G. Geesey, and K. J. Cheng.** 1978. How Bacteria Stick. Scientific American **238:**86.
48. **Cox, W. G., M. P. Beaudet, C. Ying, S. Yue, X. Chen, V. L. Singer, J. M. Beechem, and R. P. Haugland.** 2001. A new class of fluorescent dyes: Alexa Fluor 555 and Alexa Fluor 647 dyes are spectrally identical to Cy3 and Cy5 dyes, yet produce much brighter DNA probes. American Journal of Human Genetics **69:**337.
49. **Danon, S. J., and A. Lee.** 2001. Enterohepatic *Helicobacter* species, p. 549-563. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
50. **De Reuse, H., and S. Skouloubris.** 2001. Nitrogen metabolism, p. 125-134. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
51. **Defernando, G. D. G., O. D. M. Fernandez, and J. A. Ordonez.** 1992. Changes in Water Activity of Selected Solid Culture Media Throughout Incubation. Food Microbiology **9:**77-82.
52. **Degnan, A. J., W. C. Sonzogni, and J. H. Standridge.** 2003. Development of a plating medium for selection of *Helicobacter pylori* from water samples. Applied and Environmental Microbiology **69:**2914-2918.
53. **Demidov, V. V., V. N. Potaman, M. D. Frank-Kamenetskii, M. Egholm, O. Buchard, S. H. Sonnichsen, and P. E. Nielsen.** 1994. Stability of peptide nucleic acids in human serum and cellular extracts. Biochemical Pharmacology **48:**1310-3.
54. **Dore, M. P., M. Bilotta, D. Vaira, A. Manca, G. Massarelli, G. Leandro, A. Atzei, G. Pisanu, D. Y. Graham, and G. Realdi.** 1999. High prevalence of *Helicobacter pylori* infection in shepherds. Digestive Diseases and Sciences **44:**1161-1164.

55. **Dore, M. P., A. R. Sepulveda, H. El-Zimaity, Y. Yamaoka, M. S. Osato, K. Mototsugu, A. M. Nieddu, G. Realdi, and D. Y. Graham.** 2001. Isolation of *Helicobacter pylori* from sheep - Implications for transmission to humans. American Journal of Gastroenterology **96:**1396-1401.
56. **Dore, M. P., A. R. Sepulveda, M. S. Osato, G. Realdi, and D. Y. Graham.** 1999. *Helicobacter pylori* in sheep milk. Lancet **354:**132-132.
57. **Drobniewski, F. A., P. G. More, and G. S. Harris.** 2000. Differentiation of *Mycobacterium tuberculosis* complex and nontuberculous mycobacterial liquid cultures by using peptide nucleic acid-fluorescence *in situ* hybridization probes. Journal of Clinical Microbiology **38:**444-447.
58. **Dunsmore, B. C., A. Jacobsen, L. Hall-Stoodley, C. J. Bass, H. M. Lappin-Scott, and P. Stoodley.** 2002. The influence of fluid shear on the structure and material properties of sulphate-reducing bacterial biofilms. Journal of Industrial Microbiology & Biotechnology **29:**347-353.
59. **ElZaatari, F. A. K., J. S. Woo, A. Badr, M. S. Osato, H. Serna, L. M. Lichtenberger, R. M. Genta, and D. Y. Graham.** 1997. Failure to isolate *Helicobacter pylori* from stray cats indicates that *H. pylori* in cats may be an anthroponosis - An animal infection with a human pathogen. Journal of Medical Microbiology **46:**372-376.
60. **Enroth, H., and L. Engstrand.** 2001. An update on *Helicobacter pylori* microbiology and infection for the new millennium. Scandinavian Journal of Infectious Diseases **33:**163-174.
61. **Enroth, H., K. Wreiber, R. Rigo, D. Risberg, A. Uribe, and L. Engstrand.** 1999. In vitro aging of *Helicobacter pylori*: Changes in morphology, intracellular composition and surface properties. *Helicobacter* **4:**7-16.
62. **Erreimi, N., N. Mouane, S. Hajarabi, Z. Alhamani, and F. Bouchta.** 1999. *Helicobacter pylori* gastritis in children. Experience with 230 gastric biopsies performed at the Rabat pediatric hospital. Annales De Pediatrie **46:**445-451.
63. **Ferguson, R. L., E. N. Buckley, and A. V. Palumbo.** 1984. Response of Marine Bacterioplankton to Differential Filtration and Confinement. Applied and Environmental Microbiology **47:**49-55.
64. **Flanigan, D., and M. Rodgers.** 2003. A method to detect viable *Helicobacter pylori* bacteria in groundwater. Acta Hydrochimica Et Hydrobiologica **31:**45-48.
65. **Fujimura, S., T. Kawamura, S. Kato, H. Tateno, and A. Watanabe.** 2002. Detection of *Helicobacter pylori* in cow's milk. Letters in Applied Microbiology **35:**504-507.
66. **Fukuda-Parr, S.** 2004. Human development report 2004: Cultural liberty in today's diverse world. United Nations Development Programme, New York.
67. **Gardner, D. J.** 1996. Application of the Lifshitz-van der Waals acid-base approach to determine wood surface tension components. Wood and Fiber Science **28:**422-428.
68. **Ge, Z. M., K. Hiratsuka, and D. E. Taylor.** 1995. Nucleotide-Sequence and Mutational Analysis Indicate That 2 *Helicobacter pylori* Genes Encode a P-Type Atpase and a Cation-Binding Protein Associated with Copper Transport. Molecular Microbiology **15:**97-106.

69. **Geldreich, E. E.** 1996. Microbial quality of water supply in distribution systems. CRC press, Boca Raton, Florida.
70. **Go, M. F., V. Kapur, D. Y. Graham, and J. M. Musser.** 1996. Population genetic analysis of *Helicobacter pylori* by multilocus enzyme electrophoresis: Extensive allelic diversity and recombinational population structure. Journal of Bacteriology **178:**3934-3938.
71. **Godoy, P., A. Artigues, C. Nuin, J. Aramburu, M. Perez, A. Dominguez, and L. Salleras.** 2002. Outbreak of gastroenteritis caused by *Campylobacter jejuni* transmitted through drinking water. Medicina Clinica **119:**695-698.
72. **Gomes, B. C., and E. C. P. De Martinis.** 2004. The significance of *Helicobacter pylori* in water, food and environmental samples. Food Control **15:**397-403.
73. **Goodarzi, F., and P. K. Mukhopadhyay.** 2000. Metals and polyaromatic hydrocarbons in the drinking water of the Sydney Basin, Nova Scotia, Canada: a preliminary assessment of their source. International Journal of Coal Geology **43:**357-372.
74. **Goodman, K. J., P. Correa, H. J. Tengana Aux, H. Ramirez, J. P. DeLany, O. Guerrero Pepinosa, M. Lopez Quinones, and T. Collazos Parra.** 1996. *Helicobacter pylori* infection in the Colombian Andes: a population-based study of transmission pathways. American Journal of Epidemiology **144:**290-299.
75. **Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. Sly, W. Mcconnell, and W. E. S. Harper.** 1989. Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* Gen-Nov as *Helicobacter pylori* Comb-Nov and *Helicobacter mustelae* Comb-Nov, Respectively. International Journal of Systematic Bacteriology **39:**397-405.
76. **Graham, D. Y., and H. M. Malaty.** 2002. Commentary: What remains to be done regarding transmission of *Helicobacter pylori*. International Journal of Epidemiology **31:**646-647.
77. **Grubel, P., J. S. Hoffman, F. K. Chong, N. A. Burstein, C. Mepani, and D. R. Cave.** 1997. Vector potential of houseflies (*Musca domestica*) for *Helicobacter pylori*. Journal of Clinical Microbiology **35:**1300-1303.
78. **Gueneau, P., J. Fuenmayor, O. C. Aristimuno, S. Cedeno, E. Baez, N. Reyes, F. Michelangeli, and M. G. Dominguez-Bello.** 2002. Are goats naturally resistant to gastric *Helicobacter* infection? Veterinary Microbiology **84:**115-121.
79. **Hachem, C. Y., J. E. Clarridge, D. G. Evans, and D. Y. Graham.** 1995. Comparison of Agar Based Media for Primary Isolation of *Helicobacter pylori*. Journal of Clinical Pathology **48:**714-716.
80. **Hall-Stoodley, L., and P. Stoodley.** 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends in Microbiology **13:**7-10.
81. **Han, S. W., D. G. Evans, F. A. K. ElZaatari, M. F. Go, and D. Graham.** 1996. The interaction of pH, bile, and *Helicobacter pylori* may explain duodenal ulcer. American Journal of Gastroenterology **91:**1135-1137.
82. **Hanninen, M. L.** 1991. Sensitivity of *Helicobacter pylori* to Different Bile-Salts. European Journal of Clinical Microbiology & Infectious Diseases **10:**515-518.
83. **Harper, C. G., Y. Feng, S. Xu, N. S. Taylor, A. Kinsel, F. E. Dewhirst, B. J. Paster, M. Greenwell, G. Levine, A. Rogers, and J. G. Fox.** 2002. *Helicobacter*

cetorum sp nov., a urease-positive *Helicobacter* species isolated from dolphins and whales. Journal of Clinical Microbiology **40:**4536-4543.

84. **Harper, C. G., S. Xu, A. B. Rogers, Y. Feng, Z. Shen, N. S. Taylor, F. E. Dewhirst, B. J. Paster, M. Miller, J. Hurley, and J. G. Fox.** 2003. Isolation and characterization of novel *Helicobacter* spp. from the gastric mucosa of harp seals *Phoca groenlandica*. Dis Aquat Organ **57:**1-9.
85. **Hassan, A. N., and J. F. Frank.** 2004. Attachment of *Escherichia coli* O157 : H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity, surface charge, and capsule production. International Journal of Food Microbiology **96:**103-109.
86. **Hazell, S. L., A. G. Harris, and M. A. Trend.** 2001. Evasion of the toxic effects of oxygen, p. 167-178. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
87. **Henriksen, T. H., A. Lia, R. Schoyen, T. Thoresen, and A. Berstad.** 2000. Assessment of optimal atmospheric conditions for growth of *Helicobacter pylori*. Eur J Clin Microbiol Infect Dis **19:**718-720.
88. **Herbarth, O., P. Krumbiegel, G. J. Fritz, M. Richter, U. Schlink, D. M. Muller, and T. Richter.** 2001. *Helicobacter pylori* prevalences and risk factors among school beginners in a German urban center and its rural county. Environmental Health Perspectives **109:**573-577.
89. **Hildebrand, P., B. M. Meyer-Wyss, S. Mossi, and C. Beglinger.** 2000. Risk among gastroenterologists of acquiring *Helicobacter pylori* infection: case-control study. British Medical Journal **321:**149-149.
90. **Hitt, E.** 2004. On the iTRAQ. Scientist **18:**43.
91. **Hoebe, C. J. P. A., H. Vennema, A. M. D. Husman, and Y. T. H. P. van Duynhoven.** 2004. Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. Journal of Infectious Diseases **189:**699-705.
92. **Hoffman, P. S., A. Goodwin, J. Johnsen, K. Magee, S. J. O. Veldhuyzen, and S. J. O. V. vanZanten.** 1996. Metabolic activities of metronidazole-sensitive and -resistant strains of *Helicobacter pylori*: Repression of pyruvate oxidoreductase and expression of isocitrate lyase activity correlate with resistance. Journal of Bacteriology **178:**4822-4829.
93. **Holden, B., M. Greetham, B. T. Croll, and J. Scutt.** 1995. The effect of changing inter process and final disinfection reagents on corrosion and biofilm growth in distribution pipes. Water Science and Technology **32:**213-220.
94. **Hopkins, R. J., P. A. Vial, C. Ferreccio, J. Ovalle, P. Prado, V. Sotomayor, R. G. Russell, S. S. Wasserman, and J. G. Morris, Jr.** 1993. Seroprevalence of *Helicobacter pylori* in Chile: vegetables may serve as one route of transmission. Journal of Infectious Diseases **168:**222-226.
95. **Horiuchi, T., T. Ohkusa, M. Watanabe, D. Kobayashi, H. Miwa, and Y. Eishi.** 2001. *Helicobacter pylori* DNA in drinking water in Japan. Microbiology and Immunology **45:**515-519.

96. **Horn, H., H. Reiff, and E. Morgenroth.** 2003. Simulation of growth and detachment in biofilm systems under defined hydrodynamic conditions. Biotechnology and Bioengineering **81:**607-617.
97. **Huang, C. T., G. A. McFeters, and P. S. Stewart.** 1996. Evaluation of physiological staining, cryoembedding and autofluorescence quenching techniques on fouling biofilms. Biofouling **9:**269.
98. **Ilboudo, D., A. Sawadogo, A. Bougouma, R. Sombie, and F. Koueta.** 1998. An epidemiologic study of *Helicobacter pylori* infection in children in Burkina Faso. Annales De Pediatrie **45:**425-429.
99. **Ilver, D., A. Arnqvist, J. Ogren, I. M. Frick, D. Kersulyte, E. T. Incecik, D. E. Berg, A. Covacci, L. Engstrand, and T. Boren.** 1998. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science **279:**373-377.
100. **Imamura, S., M. Kita, Y. Yamaoka, T. Yamamoto, A. Ishimaru, H. Konishi, N. Wakabayashi, S. Mitsufuji, T. Okanoue, and J. Imanishi.** 2003. Vector potential of cockroaches for *Helicobacter pylori* infection. American Journal of Gastroenterology **98:**1500-1503.
101. **Ishii, N., M. Robert, Y. Nakayama, A. Kanai, and M. Tomita.** 2004. Toward large-scale modeling of the microbial cell for computer simulation. Journal of Biotechnology **113:**281-294.
102. **Israel, D. A., A. S. Lou, and M. J. Blaser.** 2000. Characteristics of *Helicobacter pylori* natural transformation. FEMS Microbiology Letters **186:**275-280.
103. **Jang, A., P. L. Bishop, S. Okabe, S. G. Lee, and I. S. Kim.** 2003. Effect of dissolved oxygen concentration on the biofilm and in situ analysis by fluorescence in situ hybridization (FISH) and microelectrodes. Water Science & Technology **47:**49-57.
104. **Jiang, X. P., and M. P. Doyle.** 1998. Effect of environmental and substrate factors on survival and growth of *Helicobacter pylori*. Journal of Food Protection **61:**929-933.
105. **Johnson, C. H., E. W. Rice, and D. J. Reasoner.** 1997. Inactivation of *Helicobacter pylori* by chlorination. Applied and Environmental Microbiology **63:**4969-4970.
106. **Kabir, S.** 2001. Detection of *Helicobacter pylori* in faeces by culture, PCR and enzyme immunoassay. Journal of Medical Microbiology **50:**1021-1029.
107. **Karita, M., S. Teramukai, and S. Matsumoto.** 2003. Risk of *Helicobacter pylori* transmission from drinking well water is higher than that from infected intrafamilial members in Japan. Digestive Diseases and Sciences **48:**1062-1067.
108. **Kearney, D. J., and C. Crump.** 2002. Domestic cats and cogs and home drinking water source as risk factors for *Helicobacter pylori* infection in the United States. Gut **51:**A31-A32.
109. **Keevil, C. W.** 2001. Continuous culture methods to study pathogens in biofilms. Methods in Enzymology **334:**104-122.
110. **Keevil, C. W.** 2001. Continuous culture models to study pathogens in biofilms. Microbial Growth in Biofilms, Pt B **337:**104-122.

111. **Keevil, C. W.** 2003. Pathogens in environmental biofilms. *In* G. Bitton (ed.), The Encyclopaedia of Environmental Microbiology. Wiley, New York.
112. **Keevil, C. W.** 2003. Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. Water Science and Technology **47:**105-116.
113. **Keevil, C. W., J. Rogers, and J. T. Walker.** 1995. Potable water biofilms. Microbiology Europe **3:**10-14.
114. **Kelly, D. B., and N. J. Hughes.** 2001. The citric acid cycle and fatty acid biosynthesis, p. 135-146. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
115. **Kelly, D. B., N. J. Hughes, and R. K. Poole.** 2001. Microaerobic physiology: aerobic respiration, anaerobic respiration and carbon dioxide metabolism, p. 113-124. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
116. **Kerr, C. J., K. S. Osborn, G. D. Robson, and P. S. Handley.** 1999. The relationship between pipe material and biofilm formation in a laboratory model system. Journal of Applied Microbiology **85:**29s-38s.
117. **Khandaker, K., K. R. Palmer, M. A. Eastwood, A. C. Scott, M. Desai, and R. J. Owen.** 1993. DNA Fingerprints of Helicobacter-Pylori from Mouth and Antrum of Patients with Chronic Ulcer Dyspepsia. Lancet **342:**751-751.
118. **Kharazmi, A., B. Giwercman, and N. Hoiby.** 1999. Robbins device in biofilm research. Biofilms **310:**207-215.
119. **Kitagawa, M., M. Natori, M. Katoh, K. Sugimoto, H. Omi, Y. Akiyama, and H. Sago.** 2001. Maternal transmission of *Helicobacter pylori* in the perinatal period. J Obstet Gynaecol Research **27:**225-230.
120. **Klein, P. D., D. Y. Graham, A. Gaillour, A. R. Opekun, and E. O. Smith.** 1991. Water Source as Risk Factor for Helicobacter-Pylori Infection in Peruvian Children. Lancet **337:**1503-1506.
121. **Kolter, R.** 2005. Surfacing views of biofilm biology. Trends in Microbiology **13:**1-2.
122. **Krishnamurthy, P., S. H. Phadnis, C. DeLoney, R. S. Rosenthal, and B. E. Dunn.** 2001. Biosynthetic pathways related to cell structure and function, p. 159-166. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
123. **Krumbiegel, P., I. Lehmann, A. Alfreider, G. J. Fritz, D. Boeckler, U. Rolle-Kampczyk, M. Richter, S. Jorks, L. Muller, M. W. Richter, and O. Herbarth.** 2004. *Helicobacter pylori* determination in non-municipal drinking water and epidemiological findings. Isotopes in Environmental and Health Studies **40:**75-80.
124. **Kusters, J. G., M. M. Gerrits, J. A. G. VanStrijp, and C. M. J. E. VandenbrouckeGrauls.** 1997. Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. Infection and Immunity **65:**3672-3679.
125. **Kuusi, M., P. Klemets, I. Miettinen, I. Laaksonen, H. Sarkkinen, M. L. Hanninen, H. Rautelin, E. Kela, and J. P. Nuorti.** 2004. An outbreak of gastroenteritis from a non-chlorinated community water supply. Journal of Epidemiology and Community Health **58:**273-277.

126. **Lancaster, C. R., and J. Simon.** 2002. Succinate:quinone oxidoreductases from epsilon-proteobacteria. Biochim Biophys Acta **1553:**84-101.
127. **Lawrence, J. R., and T. R. Neu.** 1999. Confocal laser scanning microscopy for analysis of microbial biofilms. Biofilms **310:**131-144.
128. **Lehtola, M. J., T. Juhna, I. T. Miettinen, T. Vartiainen, and P. J. Martikainen.** 2004. Formation of biofilms in drinking water distribution networks, a case study in two cities in Finland and Latvia. Journal of Industrial Microbiology & Biotechnology **31:**489-494.
129. **Li, Y., W. A. Dick, and O. H. Tuovinen.** 2004. Fluorescence microscopy for visualization of soil microorganisms - a review. Biology and Fertility of Soils **39:**301-311.
130. **Lin, D. B., W. T. Nieh, H. M. Wang, M. W. Hsiao, U. P. Ling, S. P. Changlai, M. S. Ho, S. L. You, and C. J. Chen.** 1999. Seroepidemiology of *Helicobacter pylori* infection among preschool children in Taiwan. American Journal of Tropical Medicine and Hygiene **61:**554-558.
131. **Lin, F. Y. H., Z. Policova, A. W. Neumann, and P. M. Sherman.** 1999. Surface hydrophobicity does not identify ulcerogenic *Helicobacter pylori* strains isolated from children and adolescents. Colloids and Surfaces B-Biointerfaces **15:**365-370.
132. **Lindo, J. F., A. E. Lyn-Sue, C. J. Palmer, M. G. Lee, P. Vogel, and R. D. Robinson.** 1999. Seroepidemiology of *Helicobacter pylori* infection in a Jamaican community. Tropical Medicine & International Health **4:**862-866.
133. **Loy, A., M. Horn, and M. Wagner.** 2003. probeBase: an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Research **31:**514-516.
134. **Luman, W.** 2002. *Helicobacter pylori* transmission: is it due to kissing? J R Coll Physicians Edinb **32:**275-279.
135. **Luman, W., A. M. Alkout, C. C. Blackwell, D. M. Weir, and K. R. Palmer.** 1996. *Helicobacter pylori* in the mouth - Negative isolation from dental plaque and saliva. European Journal of Gastroenterology & Hepatology **8:**11-14.
136. **Luman, W., Y. Zhao, H. S. Ng, and K. L. Ling.** 2002. *Helicobacter pylori* infection is unlikely to be transmitted between partners: evidence from genotypic study in partners of infected patients. European Journal of Gastroenterology & Hepatology **14:**521-528.
137. **Lunet, N., and H. Barros.** 2003. *Helicobacter pylori* infection and gastric cancer: Facing the enigmas. International Journal of Cancer **106:**953-960.
138. **Lyra, A. C., G. Santana, N. Santana, A. Silvany-Neto, E. Magalhaes, E. M. Pereira, R. Mascarenhas, M. C. Lyra, A. Veiga, K. Ferreira, S. Zaterka, and L. G. Lyra.** 2003. Seroprevalence and risk factors associated with *Helicobacter pylori* infection in blood donors in Salvador, Northeast-Brazil. Brazilian Journal of Infectious Diseases **7:**339-745.
139. **Ma, F. J., W. Y. Zhao, M. Kudo, K. Aoki, and J. Misumi.** 2002. Inhibition of vacuolation toxin activity of *Helicobacter pylori* by iodine, nitrite and potentiation by sodium chloride, sterigmatocystin and fluoride. Toxicology in Vitro **16:**531-537.
140. **Ma, J. L., W. C. You, M. H. Gail, L. Zhang, W. J. Blot, Y. S. Chang, J. Jiang, W. D. Liu, Y. R. Hu, L. M. Brown, G. W. Xu, and J. F. Fraumeni, Jr.** 1998.

Helicobacter pylori infection and mode of transmission in a population at high risk of stomach cancer. International Journal of Epidemiology **27:**570-573.

141. **Mackay, W. G., L. T. Gribbon, M. R. Barer, and D. C. Reid.** 1999. Biofilms in drinking water systems: a possible reservoir for *Helicobacter pylori*. Journal of Applied Microbiology **85:**52s-59s.
142. **Madigan, M., J. Martinko, and J. Parker.** 1997. Brock Biology of Microorganisms. Prentice-Hall International, London.
143. **Maier, D., M. Maier, B. Lloyd, and I. P. Toms.** 1997. Remobilization of polynuclear aromatic hydrocarbons from the coal tar linings of water mains. Ozone Science & Engineering **18:**517-519.
144. **Manz, W., K. Wendt-Potthoff, T. R. Neu, U. Szewzyk, and J. R. Lawrence.** 1999. Phylogenetic Composition, Spatial Structure, and Dynamics of Lotic Bacterial Biofilms Investigated by Fluorescent in Situ Hybridization and Confocal Laser Scanning Microscopy. Microb Ecology **37:**225-237.
145. **Marais, A., G. L. Mendz, S. L. Hazell, and F. Megraud.** 1999. Metabolism and genetics of *Helicobacter pylori*: the genome era. Microbiol Mol Biol Rev **63:**642-674.
146. **Marshall, B. J.** 2001. One hundred years of discovery and rediscovery of *Helicobacter pylori* and its association with peptic ulcer disease, p. 19-24. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
147. **Marshall, B. J., and C. S. Goodwin.** 1987. Revised Nomenclature of *Campylobacter pyloridis*. International Journal of Systematic Bacteriology **37:**68.
148. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1:**1311-5.
149. **Massa, S., M. Caruso, F. Trovatelli, and M. Tosques.** 1998. Comparison of plate count agar and R2A medium for enumeration of heterotrophic bacteria in natural mineral water. World Journal of Microbiology & Biotechnology **14:**727-730.
150. **McClaine, J. W., and R. M. Ford.** 2002. Characterizing the adhesion of motile and nonmotile *Escherichia coli* to a glass surface using a parallel-plate flow chamber. Biotechnol Bioeng **78:**179-189.
151. **Mcfeters, G. A., F. P. P. Yu, B. H. Pyle, and P. S. Stewart.** 1995. Physiological assessment of bacteria using fluorochromes. Journal of Microbiological Methods **21:**1-13.
152. **McGinnis, S., and T. L. Madden.** 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Research **32:**W20-W25.
153. **McLoughlin, R., I. Racz, M. Buckley, H. J. O'Connor, and C. O'Morain.** 2004. Therapy of *Helicobacter pylori*. *Helicobacter* **9 Suppl 1:**42-8.
154. **Megraud, F.** 2003. When and how does *Helicobacter pylori* infection occur? Gastroenterologie Clinique Et Biologique **27:**374-379.
155. **Melo, L. F., and T. R. Bott.** 1997. Biofouling in water systems. Experimental Thermal and Fluid Science **14:**375-381.
156. **Mendz, G. L.** 2001. Nucleotide metabolism, p. 147-158. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.

157. **Michalet, X., F. F. Pinaud, L. A. Bentolila, J. M. Tsay, S. Doose, J. J. Li, G. Sundaresan, A. M. Wu, S. S. Gambhir, and S. Weiss.** 2005. Quantum dots for live cells, in vivo imaging, and diagnostics. Science **307:**538-544.
158. **Mitchell, H. M.** 2001. Epidemiology of infection, p. 7-18. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
159. **Mitchell, H. M., Y. Y. Li, P. J. Hu, S. L. Hazell, G. G. Du, D. J. Byrne, and A. Lee.** 1992. The Susceptibility of *Helicobacter pylori* to Bile May Be an Obstacle to Fecal Transmission. European Journal of Gastroenterology & Hepatology **4:**S79-S83.
160. **Moayyedi, P., and R. H. Hunt.** 2004. *Helicobacter pylori* public health implications. *Helicobacter* **9 Suppl 1:**67-72.
161. **Momba, M. N. B., R. Kfir, S. N. Venter, and T. E. Cloete.** 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. Water Sa **26:**59-66.
162. **Momba, M. N. B., and N. Makala.** 2004. Comparing the effect of various pipe materials on biofilm formation in chlorinated and combined chlorine-chloraminated water systems. Water Sa **30:**175-182.
163. **Moreno, Y., M. A. Ferrus, J. L. Alonso, A. Jimenez, and J. Hernandez.** 2003. Use of fluorescent in situ hybridization to evidence the presence of *Helicobacter pylori* in water. Water Research **37:**2251-2256.
164. **Moshkowitz, M., A. Gorea, N. Arber, F. Konikoff, S. Berger, and T. Gilat.** 1994. Morphological Transformation of *Helicobacter pylori* During Prolonged Incubation - Association with Decreased Acid Resistance. Journal of Clinical Pathology **47:**172-174.
165. **Ndip, R. N., A. E. Malange, J. F. T. Akoachere, W. G. MacKay, V. P. K. Titanji, and L. T. Weaver.** 2004. *Helicobacter pylori* antigens in the faeces of asymptomatic children in the Buea and Limbe health districts of Cameroon: a pilot study. Tropical Medicine & International Health **9:**1036-1040.
166. **Neiger, R., and K. W. Simpson.** 2000. *Helicobacter* infection in dogs and cats: Facts and fiction. Journal of Veterinary Internal Medicine **14:**125-133.
167. **Neumann, M., and D. Gabel.** 2002. Simple method for reduction of autofluorescence in fluorescence microscopy. Journal of Histochemistry & Cytochemistry **50:**437-439.
168. **Nielsen, P. E., M. Egholm, R. H. Berg, and O. Buchardt.** 1991. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science **254:**1497-500.
169. **Nielsen, P. H., M. A. de Muro, and J. L. Nielsen.** 2000. Studies on the in situ physiology of *Thiothrix* spp. present in activated sludge. Environmental Microbiology **2:**389-398.
170. **Nilsson, H. O., J. Blom, W. Abu-Al-Soud, A. A. Ljungh, L. P. Andersen, and T. Wadstrom.** 2002. Effect of cold starvation, acid stress, and nutrients on metabolic activity of *Helicobacter pylori*. Applied and Environmental Microbiology **68:**11-19.
171. **Nogueira, R., L. F. Melo, U. Purkhold, S. Wuertz, and M. Wagner.** 2002. Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of

hydraulic retention time and the presence of organic carbon. Water Research **36:**469-481.

172. **Notarnicola, M., F. Russo, A. Cavallini, M. Bianco, E. Jirillo, S. Pece, C. Leoci, G. diMatteo, and A. diLeo.** 1996. PCR identification of *Helicobacter pylori* DNA in faeces from patients with gastroduodenal pathology. Medical Science Research **24:**785-787.
173. **Nurgalieva, Z. Z., H. M. Malaty, D. Y. Graham, R. Almuchambetova, A. Machmudova, D. Kapsultanova, M. S. Osato, F. B. Hollinger, and A. Zhangabylov.** 2002. *Helicobacter pylori* infection in Kazakhstan: Effect of water source and household hygiene. American Journal of Tropical Medicine and Hygiene **67:**201-206.
174. **Nystrom, T.** 2001. Not quite dead enough: on bacterial life, culturability, senescence, and death. Archives of Microbiology **176:**159-164.
175. **Ohno, A., N. Kato, K. Yamada, and K. Yamaguchi.** 2003. Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. Applied and Environmental Microbiology **69:**2540-2547.
176. **Okabe, S., T. Itoh, H. Satoh, and Y. Watanabe.** 1999. Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. Appl Environ Microbiol **65:**5107-16.
177. **Ollos, P. J., P. M. Huck, and R. M. Slawson.** 2003. Factors affecting biofilm accumulation in model distribution systems. Journal American Water Works Association **95:**87.
178. **Olmos, J. A., H. Rios, R. Higa, and A. H. E. Study.** 2000. Prevalence of *Helicobacter pylori* infection in Argentina - Results of a nationwide epidemiologic study. Journal of Clinical Gastroenterology **31:**33-37.
179. **Olson, J. W., and R. J. Maier.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298:**1788-1790.
180. **Ong, S. P., and A. Duggan.** 2004. Eradication of *Helicobacter pylori* in clinical situations. Clinical Exp Med **4:**30-38.
181. **Orum, H., P. E. Nielsen, M. Jorgensen, C. Larsson, C. Stanley, and T. Koch.** 1995. Sequence-Specific Purification of Nucleic-Acids by Pna-Controlled Hybrid Selection. Biotechniques **19:**472-480.
182. **Osato, M. S., K. Ayub, H. H. Le, R. Reddy, and D. Y. Graham.** 1998. Houseflies are an unlikely reservoir or vector for *Helicobacter pylori*. Journal of Clinical Microbiology **36:**2786-2788.
183. **Osato, M. S., S. G. Reddy, and D. Y. Graham.** 1999. Osmotic effect of honey on growth and viability of *Helicobacter pylori*. Digestive Diseases Science **44:**462-464.
184. **Palestrant, D., Z. E. Holzknecht, B. H. Collins, W. Parker, S. E. Miller, and R. R. Bollinger.** 2004. Microbial biofilms in the gut: Visualization by electron microscopy and by acridine orange staining. Ultrastructural Pathology **28:**23-27.
185. **Papiez, D., P. C. Konturek, W. Bielanski, M. Plonka, M. Dobrzanska, A. Kaminska, U. Szczyrk, A. Bochenek, and E. Wierzchos.** 2003. Prevalence of *Helicobacter pylori* infection in Polish shepherds and their families. Digestive Liver Diseases **35:**10-15.

186. **Park, S. R., W. G. Mackay, and D. C. Reid.** 2001. *Helicobacter* sp. recovered from drinking water biofilm sampled from a water distribution system. Water Research **35:**1624-1626.
187. **Parsonnet, J., H. Shmuely, and T. Haggerty.** 1999. Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. Jama-Journal of the American Medical Association **282:**2240-2245.
188. **Pedersen, K.** 1990. Biofilm development on stainless-steel and PVC surfaces in drinking-water. Water Research **24:**239-243.
189. **Percival, S. L., J. S. Knapp, D. S. Wales, and R. G. J. Edyvean.** 1999. Biofilm development in potable quality water. Biofouling **13:**259.
190. **Percival, S. L., J. S. Knapp, D. S. Wales, and R. G. J. Edyvean.** 1999. The effect of turbulent flow and surface roughness on biofilm formation in drinking water. Journal of Industrial Microbiology & Biotechnology **22:**152-159.
191. **Pereira, M. O., P. Morin, M. J. Vieira, and L. F. Melo.** 2002. A versatile reactor for continuous monitoring of biofilm properties in laboratory and industrial conditions. Letters in Applied Microbiology **34:**22-26.
192. **Perez-Perez, G. I., D. Rothenbacher, and H. Brenner.** 2004. Epidemiology of *Helicobacter pylori* Infection. *Helicobacter* **9 Suppl 1:**1-6.
193. **Perry-O'Keefe, H., S. Rigby, K. Oliveira, D. Sorensen, H. Stender, J. Coull, and J. J. Hyldig-Nielsen.** 2001. Identification of indicator microorganisms using a standardized PNA FISH method. Journal of Microbiological Methods **47:**281-292.
194. **Piccolomini, R., G. DeBonaventura, D. Festi, G. Catamo, F. Laterza, and M. Neri.** 1997. Optimal combination of media for primary isolation of *Helicobacter pylori* from gastric biopsy specimens. Journal of Clinical Microbiology **35:**1541-1544.
195. **Poms, R. E., and S. R. Tatini.** 2001. Survival of *Helicobacter pylori* in ready-to-eat foods at 4 degrees C. International Journal of Food Microbiology **63:**281-286.
196. **Pounder, R. E., and D. Ng.** 1995. The Prevalence of *Helicobacter pylori* Infection in Different Countries. Alimentary Pharmacology & Therapeutics **9:**33-39.
197. **Pride, D. T., and M. J. Blaser.** 2002. Concerted evolution between duplicated genetic elements in *Helicobacter pylori*. Journal of Molecular Biology **316:**629-642.
198. **Prinz, C., N. Hafsi, and P. Voland.** 2003. *Helicobacter pylori* virulence factors and the host immune response: implications for therapeutic vaccination. Trends in Microbiology **11:**134-138.
199. **Reasoner, D. J., and E. E. Geldreich.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. Applied and Environmental Microbiology **49:**1-7.
200. **Reid, G., P. L. Cuperus, A. W. Bruce, H. C. Vandermei, L. Tomeczek, A. H. Khoury, and H. J. Busscher.** 1992. Comparison of Contact Angles and Adhesion to Hexadecane of Urogenital, Dairy, and Poultry Lactobacilli - Effect of Serial Culture Passages. Applied and Environmental Microbiology **58:**1549-1553.
201. **Rodgers, A.** 2002. Costs of illness. *In* WHO (ed.), The World health report : 2002 : Reducing risks, promoting healthy life, Geneva.

202. **Rodrigues, M. N., D. M. M. Queiroz, J. G. B. Filho, L. K. Pontes, R. T. Rodrigues, and L. L. B. C. Braga.** 2004. Prevalence of *Helicobacter pylori* infection in children from an urban community in north-east Brazil and risk factors for infection. European Journal of Gastroenterology & Hepatology **16:**201-205.
203. **Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil.** 1994. Influence of plumbing materials on biofilm formation and growth of *Legionella-pneumophila* in potable water-systems. Applied and Environmental Microbiology **60:**1842-1851.
204. **Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil.** 1994. Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella-pneumophila* in a model potable water-system containing complex microbial-flora. Applied and Environmental Microbiology **60:**1585-1592.
205. **Rogers, J., and C. W. Keevil.** 1992. Immunogold and Fluorescein Immunolabeling of *Legionella-Pneumophila* within an Aquatic Biofilm Visualized by Using Episcopic Differential Interference Contrast Microscopy. Applied and Environmental Microbiology **58:**2326-2330.
206. **Rolle-Kampczyk, U. E., G. J. Fritz, U. Diez, I. Lehmann, M. Richter, and O. Herbarth.** 2004. Well water - one source of *Helicobacter pylori* colonization. International Journal of Hygiene and Environmental Health **207:**363-368.
207. **Roma-Giannikou, E., A. Karameris, B. Balatsos, J. Panayiotou, Z. Manika, C. Van-Vliet, T. Rokkas, N. Skandalis, and C. Kattamis.** 2003. Intrafamilial Spread of *Helicobacter pylori*: A Genetic Analysis. *Helicobacter* **8:**15-20.
208. **Rothenbacher, D., G. Bode, G. Berg, U. Knayer, T. Gonser, G. Adler, and H. Brenner.** 1999. *Helicobacter pylori* among preschool children and their parents: Evidence of parent-child transmission. Journal of Infectious Diseases **179:**398-402.
209. **Ruggiero, P., A. Alloueche, R. Rappuoli, and G. Del Giudice.** 2004. Models for bacterial infectious diseases: *Helicobacter pylori*. Drug Discovery Today: Disease Models **1:**95-100.
210. **Russmann, H., V. A. J. Kempf, S. Koletzko, J. Heesemann, and I. B. Autenrieth.** 2001. Comparison of fluorescent in situ hybridization and conventional culturing for detection of *Helicobacter pylori* in gastric biopsy specimens. Journal of Clinical Microbiology **39:**304-308.
211. **Russo, A., M. Eboli, P. Pizzetti, G. Di Felice, F. Ravagnani, P. Spinelli, A. M. Hotz, P. Notti, G. Maconi, S. Franceschi, D. Ferrari, and L. Bertario.** 1999. Determinants of *Helicobacter pylori* seroprevalence among Italian blood donors. European Journal of Gastroenterology & Hepatology **11:**867-873.
212. **Saby, S., I. Sibille, L. Mathieu, J. L. Paquin, and J. C. Block.** 1997. Influence of water chlorination on the counting of bacteria with DAPI (4',6-diamidino-2-phenylindole). Applied and Environmental Microbiology **63:**1564-1569.
213. **Saito, N., K. Konishi, F. Sato, M. Kato, H. Takeda, T. Sugiyama, and M. Asaka.** 2003. Plural transformation-processes from spiral to coccoid *Helicobacter pylori* and its viability. Journal of Infection **46:**49-55.

214. **Salaun, L., B. Linz, S. Suerbaum, and N. J. Saunders.** 2004. The diversity within an expanded and redefined repertoire of phase-variable genes in *Helicobacter pylori*. Microbiology-SGM **150:**817-830.
215. **Salvadori, M. I., A. X. Garg, J. Howard, W. F. Clark, and D. G. Matsell.** 2002. An outbreak of *E. coli* 0157 : 117 infections due to a contaminated municipal water supply in rural Ontario - Short-term outcome of children developing HUS. Journal of the American Society of Nephrology **13:**662a-662a.
216. **Santo Domingo, J. W., S. Harmon, and J. Bennett.** 2000. Survival of Salmonella species in river water. Curr Microbiol **40:**409-17.
217. **Schauer, D. B.** 2001. Enterohepatic *Helicobacter* species, p. 533-548. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM press, Washington, D. C.
218. **Scoarughi, G. L., C. Cimmino, and P. Donini.** 1999. *Helicobacter pylori*: a eubacterium lacking the stringent response. Journal of Bacteriology **181:**552-555.
219. **Shahamat, M., U. Mai, C. Paszkokolva, M. Kessel, and R. R. Colwell.** 1993. Use of Autoradiography to Assess Viability of *Helicobacter pylori* in Water. Applied and Environmental Microbiology **59:**1231-1235.
220. **Sharma, P. K., and K. H. Rao.** 2002. Analysis of different approaches for evaluation of surface energy of microbial cells by contact angle goniometry. Advances in Colloid and Interface Science **98:**341-463.
221. **She, F. F., J. Y. Lin, J. Y. Liu, C. Huang, and D. H. Su.** 2003. Virulence of water-induced coccoid *Helicobacter pylori* and its experimental infection in mice. World Journal of Gastroenterology **9:**516-520.
222. **Sherman, P. M.** 2004. Appropriate strategies for testing and treating *Helicobacter pylori* in children: when and how? American Journal of Medicine **117 Suppl 5A:**30S-35S.
223. **Shleeva, M., G. V. Mukamolova, M. Young, H. D. Williams, and A. S. Kaprelyants.** 2004. Formation of 'non-culturable' cells of *Mycobacterium smegmatis* in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. Microbiology-SGM **150:**1687-1697.
224. **Simoes, M., M. O. Pereira, and M. J. Vieira.** 2003. Monitoring the effects of biocide treatment of *Pseudomonas fluorescens* biofilms formed under different flow regimes. Water Science and Technology **47:**217-223.
225. **Smith, A., A. B. Price, J. Dolby, and J. Levi.** 1984. *Campylobacter Pyloridis* in Peptic-Ulcer Disease - Pathogen or Opportunist. Gut **25:**1136-1137.
226. **Solnick, J. V., and D. B. Schauer.** 2001. Emergence of diverse Helicobacter species in the pathogenesis of gastric and enterohepatic diseases. Clinical Microbiology Reviews **14:**59-69.
227. **Speranza, G., G. Gottardi, C. Pederzolli, L. Lunelli, R. Canteri, L. Pasquardini, E. Carli, A. Lui, D. Maniglio, M. Brugnara, and M. Anderle.** 2004. Role of chemical interactions in bacterial adhesion to polymer surfaces. Biomaterials **25:**2029-2037.
228. **Stark, R. M., G. J. Gerwig, R. S. Pitman, L. F. Potts, N. A. Williams, J. Greenman, I. P. Weinzweig, T. R. Hirst, and M. R. Millar.** 1999. Biofilm formation by *Helicobacter pylori*. Letters Applied Microbiology **28:**121-126.

229. **Stehrgreen, J. K., C. Nicholls, S. Mcewan, A. Payne, and P. Mitchell.** 1991. Waterborne Outbreak of *Campylobacter-Jejuni* in Christchurch - the Importance of a Combined Epidemiologic and Microbiologic Investigation. New Zealand Medical Journal **104:**356-358.
230. **Stelzer, W., J. Jacob, and E. Schulze.** 1991. Environmental Aspects of Campylobacter Infections. Zentralblatt Fur Mikrobiologie **146:**3-15.
231. **Stender, H., M. Fiandaca, J. J. Hyldig-Nielsen, and J. Coull.** 2002. PNA for rapid microbiology. Journal of Microbiological Methods **48:**1-17.
232. **Stevenson, B. S., S. A. Eichorst, J. T. Wertz, T. M. Schmidt, and J. A. Breznak.** 2004. New strategies for cultivation and detection of previously uncultured microbes. Applied and Environmental Microbiology **70:**4748-4755.
233. **Stevenson, T. H., L. M. Lucia, and G. R. Acuff.** 2000. Development of a selective medium for isolation of *Helicobacter pylori* from cattle and beef samples. Applied and Environmental Microbiology **66:**723-727.
234. **Tatsuguchi, A., K. Miyake, K. Gudis, S. Futagami, T. Tsukui, K. Wada, T. Kishida, Y. Fukuda, Y. Sugisaki, and C. Sakamoto.** 2004. Effect of *Helicobacter pylori* infection on ghrelin expression in human gastric mucosa. American Journal of Gastroenterology **99:**2121-2127.
235. **Thomas, W. E., E. Trintchina, M. Forero, V. Vogel, and E. V. Sokurenko.** 2002. Bacterial adhesion to target cells enhanced by shear force. Cell **109:**913-923.
236. **Thomsen, T. R., B. V. Kjellerup, J. L. Nielsen, P. Hugenholtz, and P. H. Nielsen.** 2002. In situ studies of the phylogeny and physiology of filamentous bacteria with attached growth. Environmental Microbiology **4:**383-391.
237. **Thoreson, A. C. E., M. B. Borre, L. P. Andersen, L. Elsborg, S. Holck, P. Conway, J. Henrichsen, J. Vuust, and K. A. Krogfelt.** 1995. Development of a Pcr-Based Technique for Detection of *Helicobacter pylori*. Fems Immunology and Medical Microbiology **10:**325-333.
238. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. X. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weldman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, J. M. Weidman, C. Fujii, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.
239. **Tominaga, K., N. Hamasaki, T. Watanabe, T. Uchida, Y. Fujiwara, O. Takaishi, K. Higuchi, T. Arakawa, E. Ishii, K. Kobayashi, I. Yano, and T. Kuroki.** 1999. Effect of culture conditions on morphological changes of *Helicobacter pylori*. Journal of Gastroenterology **34 Suppl 11:**28-31.
240. **Torres, M. A., D. J. Passaro, J. Watanabe, J. Parsonnet, P. Small, J. Miyagui, C. Rodriquez, M. Astete, and R. H. Gilman.** 2003. No association between *Helicobacter pylori* and *Mycobacterium tuberculosis* infections among

gastrointestinal clinic attendees in Lima, Peru. Epidemiology and Infection **130:**87-91.

241. **Trebesius, K., K. Panthel, S. Strobel, K. Vogt, G. Faller, T. Kirchner, M. Kist, J. Heesemann, and R. Haas.** 2000. Rapid and specific detection of *Helicobacter pylori* macrolide resistance in gastric tissue by fluorescent in situ hybridisation. Gut **46:**608-614.
242. **Tsugane, S., S. Sasazuki, M. Kobayashi, S. Sasaki, and J. S. Grp.** 2004. Salt and salted food intake and subsequent risk of gastric cancer among middle-aged Japanese men and women. British Journal of Cancer **90:**128-134.
243. **Turutoglu, H., and S. Mudul.** 2002. Investigation of *Helicobacter pylori* in raw sheep milk samples. J Vet Med B Infect Dis Vet Public Health **49:**308-309.
244. **Tytgat, G. N. J.** 1995. Endoscopic Transmission of Helicobacter-Pylori. Alimentary Pharmacology & Therapeutics **9:**105-110.
245. **van der Mei, H. C., R. Bos, and H. J. Busscher.** 1998. A reference guide to microbial cell surface hydrophobicity based on contact angles. Colloids and Surfaces B-Biointerfaces **11:**213-221.
246. **Van der Wende, E., W. G. Characklis, and D. B. Smith.** 1989. Biofilms and bacterial drinking water quality. Water Research **23:**1313-1322.
247. **Van Horn, K., and C. Toth.** 1999. Evaluation of the AnaeroPack Campylo system for growth of microaerophilic bacteria. Journal of Clinical Microbiology **37:**2376-2377.
248. **van Oss, C. J., M. K. Chaudhury, and R. J. Good.** 1987. Monopolar Surfaces. Advances in Colloid and Interface Science **28:**35-64.
249. **van Oss, C. J., and R. F. Giese.** 1995. The Hydrophilicity and Hydrophobicity of Clay-Minerals. Clays and Clay Minerals **43:**474-477.
250. **Vanloosdrecht, M. C. M., J. Lyklema, W. Norde, and A. J. B. Zehnder.** 1989. Bacterial Adhesion - a Physicochemical Approach. Microbial Ecology **17:**1-15.
251. **Velazquez, M., and J. M. Feirtag.** 1999. *Helicobacter pylori*: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. International Journal of Food Microbiology **53:**95-104.
252. **Vinette, K., K. Gibney, R. Proujansky, and P. Fawcett.** 2002. Growth of *Helicobacter pylori* in a long spiral form does not alter expression of immunodominant proteins. BMC Microbiology **2:**1-9.
253. **Vogler, E. A.** 1998. Structure and reactivity of water at biomaterial surfaces. Advances in Colloid and Interface Science **74:**69-117.
254. **Walker, J. T., D. Wagner, W. Fischer, and C. W. Keevil.** 1994. Rapid Detection of Biofilm on Corroded Copper Pipes. Biofouling **8:**55-63.
255. **Wang, G., and M. P. Doyle.** 1998. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in water. Journal of Food Protection **61:**662-667.
256. **Wang, K. Y., S. N. Li, C. S. Liu, D. S. Perng, Y. C. Su, D. C. Wu, C. M. Jan, C. H. Lai, T. N. Wang, and W. M. Wang.** 2004. Effects of ingesting *Lactobacillus*- and *Bifidobacterium*-containing yogurt in subjects-with colonized *Helicobacter pylori*. American Journal of Clinical Nutrition **80:**737-741.

257. **Warren, T. M., V. Williams, and M. Fletcher.** 1992. Influence of Solid-Surface, Adhesive Ability, and Inoculum Size on Bacterial-Colonization in Microcosm Studies. Applied and Environmental Microbiology **58:**2954-2959.
258. **Wasche, S., H. Horn, and D. C. Hempel.** 2002. Influence of growth conditions on biofilm development and mass transfer at the bulk/biofilm interface. Water Research **36:**4775-4784.
259. **Watson, C. L., R. J. Owen, B. Said, S. Lai, J. V. Lee, S. Surman-Lee, and G. Nichols.** 2004. Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. Journal of Applied Microbiology **97:**690-698.
260. **Wendakoon, C. N., and L. Ozimek.** 2002. Inhibition of *Helicobacter pylori* growth by *Lactobacillus casei* in fermented milk. Milchwissenschaft-Milk Science International **57:**506-509.
261. **West, A. P., M. R. Millar, and D. S. Tompkins.** 1990. Survival of *Helicobacter pylori* in Water and Saline. Journal of Clinical Pathology **43:**609-609.
262. **Wingender, J., and H. C. Flemming.** 2004. Contamination potential of drinking water distribution network biofilms. Water Science and Technology **49:**277-286.
263. **Winiecka-Krusnell, J., K. Wreiber, A. Von Euler, L. Engstrand, and E. Linder.** 2002. Free-living amoebae promote growth and survival of *Helicobacter pylori*. Scandinavian Journal of Infectious Diseases **34:**253-256.
264. **Won, Y. S., J. H. Yoon, C. H. Lee, B. H. Kim, B. H. Hyun, and Y. K. Choi.** 2002. *Helicobacter muricola* sp nov., a novel Helicobacter species isolated from the ceca and feces of Korean wild mouse (Mus musculus molossinus). FEMS Microbiology Letters **209:**45-51.
265. **Wong, H. C., P. L. Wang, S. Y. Chen, and S. W. Chiu.** 2004. Resuscitation of viable but non-culturable *Vibrio parahaemolyticus* in a minimum salt medium. FEMS Microbiology Letters **233:**269-275.
266. **Wuyts, J., G. Perriere, and Y. V. de Peer.** 2004. The European ribosomal RNA database. Nucleic Acids Research **32:**D101-D103.
267. **Yamashita, Y., T. Fujisawa, A. Kimura, and H. Kato.** 2001. Epidemiology of *Helicobacter pylori* infection in children: A serologic study of the Kyushu region in Japan. Pediatrics International **43:**4-7.
268. **Zacheus, O. M., E. K. Iivanainen, T. K. Nissinen, M. J. Lehtola, and P. J. Martikainen.** 2000. Bacterial biofilm formation on polyvinyl chloride, polyethylene and stainless steel exposed to ozonated water. Water Research **34:**63-70.
269. **Zullo, A., C. Hassan, S. M. A. Campo, and S. Morini.** 2004. Evolving therapy for *Helicobacter pylori* infection. Expert Opinion on Therapeutic Patents **14:**1453-1464.